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THE CELLULAR MECHANISM OF RECOVERY AFTER TREATMENT WITH PENICILLIN

I. Subacute Bacterial Endocarditis

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St. Louis, Mo.

THE efficacy of a therapeutic agent may be evaluated in a number of different ways. The usual method is to observe the effect on the clinical course of the disease and to determine the per cent of patients who develop sequelae and the mortality rate with and without the agent. In this paper a little used but exceedingly valuable method of evaluation will be presented—the study of tissues from patients who have been treated with some therapeutic agent and subsequently die of the primary disease or of a complication.

This approach has the advantage of demonstrating the cellular mechanism by which a therapeutic agent facilitates healing. In this manner more may be learned of the nature of the disease, how progress of it is halted, and how repair is accomplished. Then the physician is in a position to employ the therapeutic agent more effectively or to reinforce the specific treatment with other agents.

BASIS OF STUDY

My interest in the process of healing after treatment with penicillin which led to the following investigation was first aroused in January, 1943. At that time the Department of Pathology at the Washington University School of Medicine performed an autopsy on a young woman, 33 years of age, who presumably had an induced abortion. On admission to the Barnes Hospital on Dec. 26, 1942, the patient stated that she had had prolonged vaginal bleeding which started a few days before her expected menstrual flow on December 19. Twenty-four hours before admission she experienced a severe shaking chill and thereafter had a fever. To summarize briefly, the physical signs and the course in the hospital were those of a staphylococcal septicemia with endocarditis. Cultures of the blood, sputum, urine, and uterine canal were positive for a hemolytic *Staphylococcus aureus*. The patient was given penicillin for five days for a total dose of 320,000 units. The blood culture became negative on the second day of treatment and remained so until death on Jan. 2, 1943, seven days after entry and five days after penicillin was first given.

The immediate cause of death was cerebral hemorrhage, probably from rupture of a mycotic aneurysm or erosion of an artery by an abscess, although neither of these could be demonstrated.

From the Department of Pathology, Washington University School of Medicine.
This investigation has been carried out under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Washington University.
Received for publication, Sept. 1, 1946.

The histologic structure of the cervix, endometrium, a small bit of retained placenta, ovary with a corpus luteum, breast, and pituitary indicated that the woman had been pregnant for approximately three months and that an interruption of the pregnancy occurred about ten to twelve days before death and was followed by an infection. The lesions of greatest interest were in the heart and the kidneys.

Three vegetations on the thickened mitral valve were not unusual in gross appearance. Microscopically they were not typical of acute bacterial endocarditis. The most conspicuous difference from the characteristic appearance was the absence of a layer of bacteria and fibrin on the surface. A culture showed a few staphylococci, and in sections stained for bacteria a few gram-positive cocci were observed.

The kidneys were enlarged, and over the surface were numerous small (1 to 2 mm. in diameter), depressed, red foci. The foci were collections of lymphocytes, plasma cells, and a few leucocytes with minimal necrosis.

Although there are other possible explanations of these observations on the heart and kidneys, it seemed at that time that both might be considered as healing or abortive variants of staphylococcal pyemia.

PLAN OF STUDY

Because of the interest this case aroused, thereafter the autopsies on patients treated with penicillin at the Barnes Hospital were studied a little more than was required by routine demands. It became apparent that a satisfactory study could not be made on the limited material of one hospital. Accordingly, an application was made to the Committee on Medical Research of the Office of Scientific Research and Development. The suggested investigation contemplated collection of histories, autopsy protocols, and tissue from all hospitals in which penicillin was used in 1943 and 1944, this investigation to be conducted under the auspices of the Committee. The proposal was authorized, and in the fall of 1944 the materials for study were secured in visits to twelve hospitals and schools, the staff of which were most cooperative.

Over 100 cases were available for study in four categories, namely, subacute bacterial endocarditis, pyemia, pneumonia, and meningitis; of these, the first, subacute bacterial endocarditis, illustrates most of the general principles and will be discussed in this paper. No review of the literature is included but only personal observations, many of which have been made previously by others.

As a control, eight cases from the files of the Department of Pathology were studied. These patients died between 1933 and 1935 and received no specific therapy.

THE NATURE OF THE VEGETATION IN ENDOCARDITIS

As the study proceeded it became increasingly clear that the observations could not be explained if the usually accepted idea of the nature of a vegetative endocarditis were accepted. In most textbooks the terms vegetation and thrombus are used interchangeably, and it is stated or implied that the vegetation is a thrombus.

In a perfectly formed vegetation of bacterial endocarditis, acute or subacute, there are three definite layers: a large central mass of necrotic, relatively acellular, fibrillated debris; a more peripheral layer of bacterial colonies; and a most peripheral layer of delicate fibrillary acellular material. There are slight differences in layers 1 and 3, but superficially they appear to be similar. If a vegetation is stained for elastic fibrils or for collagen, fragments of these are seen in layer 1 but not in layer 3. Further, stains for fibrin show a network in both, but in layer 1 the threads of fibrin constitute a small part of the total mass.

Since elastic and collagen fibrils are observed regularly in the central core of the vegetation, which constitutes three-quarters to seven-eighths of the total, it logically follows that this part is necrotic tissue and not thrombus. Careful study of the pattern of the fibrillary structure further confirms this. The whorls are identical with those present in the valve of chronic endocarditis.

Discussion of the implications of this conclusion on the pathogenesis and postulated growth of vegetations will be postponed until presentation of the observations on healing of subacute bacterial endocarditis.

EFFECT OF TREATMENT WITH PENICILLIN ON SUBACUTE BACTERIAL ENDOCARDITIS

From the twenty-two cases studied, five have been selected which illustrate the essential features.

CASE 1.—Barnes Hospital Autopsy No. 11,617. A 56-year-old white woman was admitted to the hospital on March 14, complaining of the following symptoms: exertional dyspnea and pain in the cardiac region since childhood; weakness, loss of weight, and chills and fever for six months; and abdominal pain and swelling of the legs for two months.

There were petechiae in the conjunctivae and on the fingers and toes. The heart was slightly enlarged. The liver and spleen were palpable and tender. Blood cultures showed *Actinomyces bovis* and alpha hemolytic streptococci. Beginning on March 15, the patient was given 40,000 units of penicillin intramuscularly every two hours for four days without benefit. Sulfadiazine was then given, and levels of 10 to 15 mg. per cent were obtained. On March 23 penicillin was begun again at 40,000 units every two hours and increased to 80,000 units every two hours on March 26. Many embolic phenomena developed and cardiac failure increased. The patient died March 30. Penicillin was last given one hour before death, and the total dose was 6,720,000 units.

The anatomic diagnosis included: chronic endocarditis of the mitral and tricuspid valves; subacute bacterial endocarditis of the tricuspid and mitral valves and posterior wall of the left atrium; infarcts of the heart, spleen, liver, and kidneys; and focal embolic glomerulonephritis.

A section of the mitral valve (Fig. 1) shows two vegetations, one sessile mass (*A*) near the line of closure and a larger pedunculated vegetation (*B*) nearer the base. In Fig. 2 (designated as area *2* in Fig. 1) is shown the entire thickness of the pedunculated vegetation. There are thin layers of tissue on each side enclosing a necrotic centrum. The granulation tissue extends outward on the surface to a point just short of the large bacterial masses (Fig. 3, designated as area *3* in Fig. 1). A higher-power view of the superficial tissue and deeper necrosis of area *2* is given in Fig. 4. The junction of the tissue with the necrotic bulbous end of the vegetation is depicted in Fig. 5.

The appearance of Figs. 1 to 5 may be explained as a proliferation of tissue through the superficial layer of fibrin and bacteria. As the capillary vessels, fibroblasts, and leucocytes advance, the bacterial colonies are destroyed. In this vegetation the process has extended about two thirds of the way from the base to the apex of the vegetation.

Fig. 2. Fig. 1.

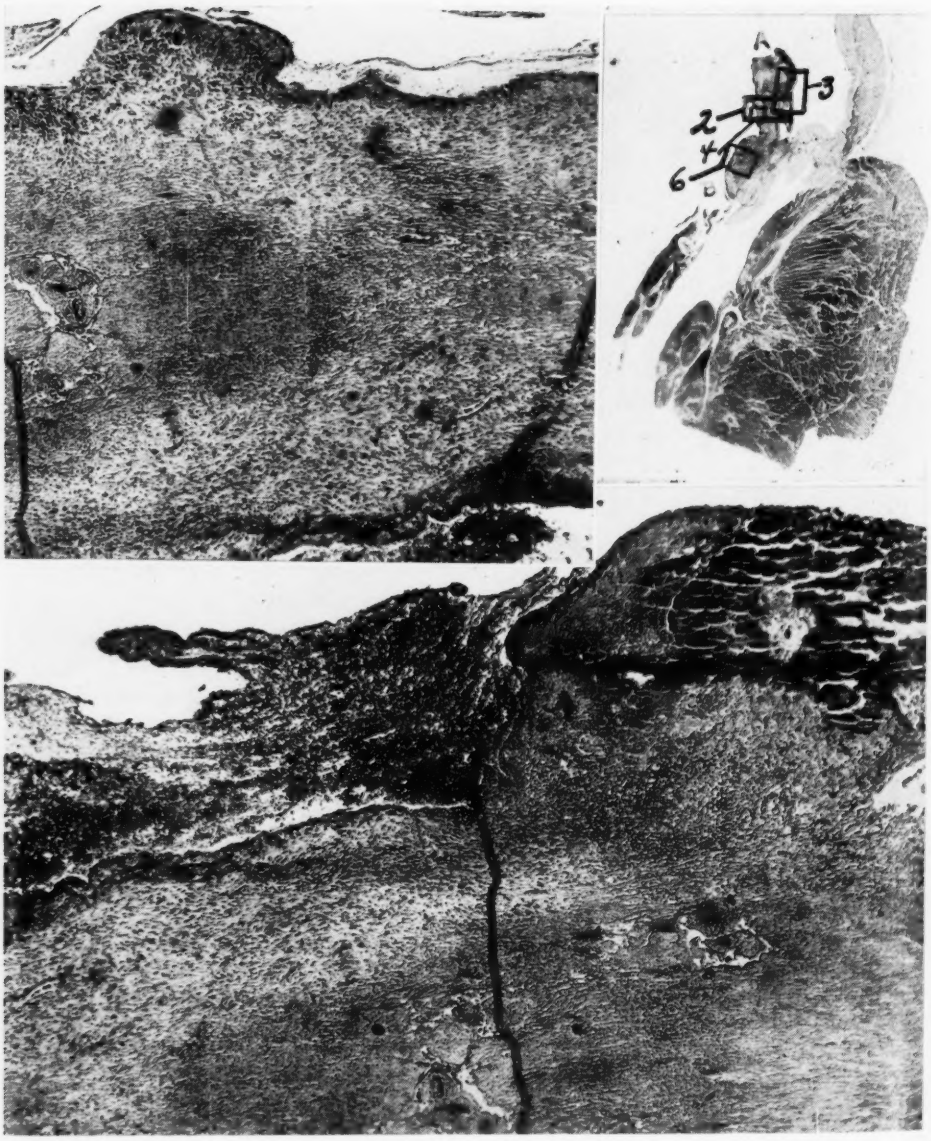

Fig. 3.

Fig. 1.—(Case 1.) Two vegetations of subacute bacterial endocarditis on the mitral valve, one pedunculated and one sessile.

Fig. 2.—(Case 1.) The stalk of the pedunculated vegetation from area 2, marked on Fig. 1. Note the tissue in the superficial parts on both sides and the central necrotic core.

Fig. 3.—(Case 1.) Higher power view of area 3, marked on Fig. 1. The tissue on the left is approaching the bacterial colonies on the right. Note that bacteria have disappeared from the part of the vegetation already organized.

Fig. 4.

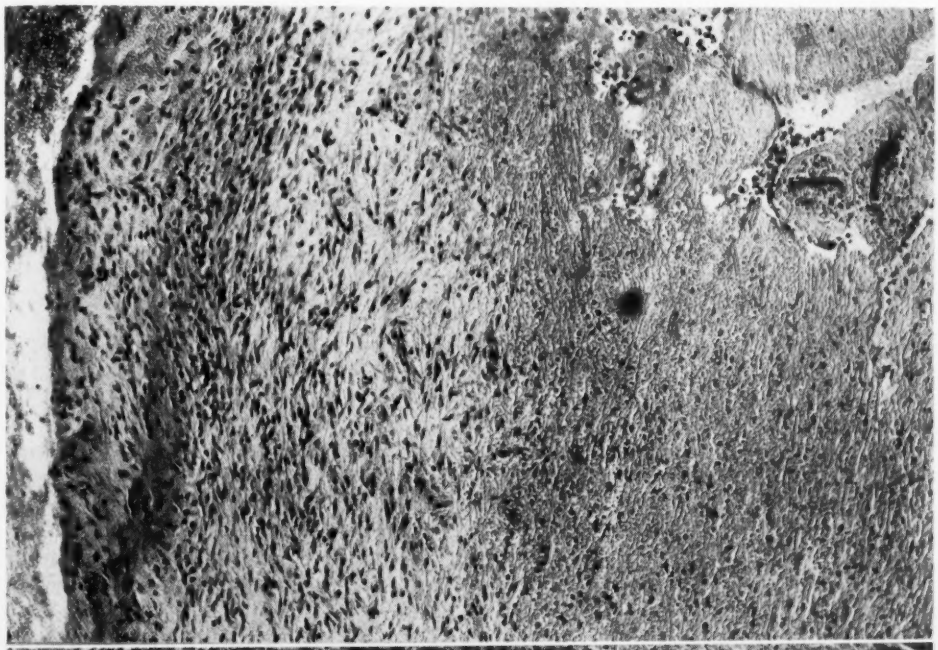

Fig. 5.

Fig. 4.—(Case 1.) Higher power photomicrograph of area 4 in Fig. 1. The superficial part of the vegetation is composed of a loose fibroblastic tissue with a distinct line of demarcation from the underlying necrotic core.

Fig. 5.—(Case 1.) Appearance of the more distal extension of the tissue in Fig. 3. As in Fig. 4, there is a sharp demarcation between the tissue and the necrotic core below.

In some instances organization may also proceed from the entire base of the vegetation. In the sessile vegetation *B*, there are successive layers of dense connective tissue, granulation tissue, lacunae filled with leucocytes, and bacterial colonies embedded in a granular debris (Fig. 6, designated *6* in Fig. 1). The lacunae (Fig. 7) probably represent former bacterial colonies which have been phagocytized and destroyed.

Case 2.—Barnes Hospital Autopsy No. 11,779. A 55-year-old white man was admitted to the hospital on May 8, 1945. Five years previously he had a left-sided hemiplegia, with almost complete recovery except for general weakness until six weeks previously when he began to notice swelling of the feet and ankles and an increasing number of petechiae. More recently he had difficulty in speaking clearly.

On May 14 a blood culture showed alpha hemolytic streptococci. Penicillin was started May 17, 40,000 units every two hours intramuscularly for fourteen days. The blood cultures remained positive. On June 1 penicillin was increased to 50,000 units every hour. The organism was then found to grow in concentrations of 20 units of penicillin per cubic centimeter. Penicillin was stopped on June 5 and begun again, 50,000 units every hour, on June 12. On June 17 test injections of 100,000 units were given intravenously every two hours and blood levels taken. Five minutes after injection the level was about 16 units per cubic centimeter. On June 18, 50,000 units every hour intramuscularly were begun again and continued until June 20, for a total of 22,610,000 units.

At that time the patient refused further injections of penicillin and signed himself out of the hospital, under protest, on June 28. He was readmitted July 22; the edema was worse, the petechiae were more abundant, there were small pustules on both hands, and he complained of shortness of breath. No chemotherapy was attempted because of evidence of extreme renal and hepatic damage. The patient developed pulmonary edema on July 31 and died on Aug. 1, 1945.

The anatomic diagnosis included: chronic endocarditis of the mitral valve; subacute bacterial endocarditis of the mitral and aortic valves and wall of the left atrium; subacute bacterial endarteritis of the ascending aorta; recent and healed infarcts of the spleen and kidneys; focal encephalomalacia of the occipital lobes and left cerebellum; focal embolic glomerulonephritis; and miliary abscesses of the myocardium.

Sections from the vegetation on the mitral valve show well the process of phagocytosis of bacteria, destruction of the colonies, and formation of lacunae. In Fig. 8 part of a colony has been destroyed and the remainder broken into smaller fragments. A large lacuna with only a small mass of bacteria at one side is depicted in Fig. 9. As the colonies are destroyed, large numbers of bacteria are seen in phagocytes (Fig. 10). The same phenomenon is present in the mural vegetations of the left atrium in this heart (Fig. 11).

The observations illustrated in Figs. 6 and 7 from Case 1 and Figs. 8 to 11 from Case 2 may be explained as phagocytosis of the bacterial colonies when living tissue reaches the proximity of the bacteria. The end result is a lacuna surrounded by necrotic vegetation or tissue.

Case 3.—Barnes Hospital Autopsy No. 11,103. A 68-year-old white woman was admitted to the hospital on January 27, complaining of fever and malaise of two and a half months' duration. The blood culture showed *Streptococcus viridans*. On February 8 the patient was transferred to another hospital and after three weeks with no improvement returned home. On March 15 the patient was readmitted to Barnes Hospital for penicillin therapy. She was disoriented and unable to speak or turn her body. There were many petechiae on the conjunctivae, face, trunk, and extremities, and the feet were swollen. The blood culture showed *Str. viridans*. Penicillin was begun on March 17, 25,000 units every two hours intramuscularly. On March 20 the blood culture was negative, but the patient failed to improve and died on March 23. Penicillin was last given five hours before death, and the total dose was 1,700,000 units.

The anatomic diagnosis included: chronic endocarditis of the mitral valve; subacute bacterial endocarditis of the mitral valve; partially healed infarcts of the spleen and kidneys; focal embolic glomerulonephritis; and subarachnoidal hemorrhage.

Fig. 6.

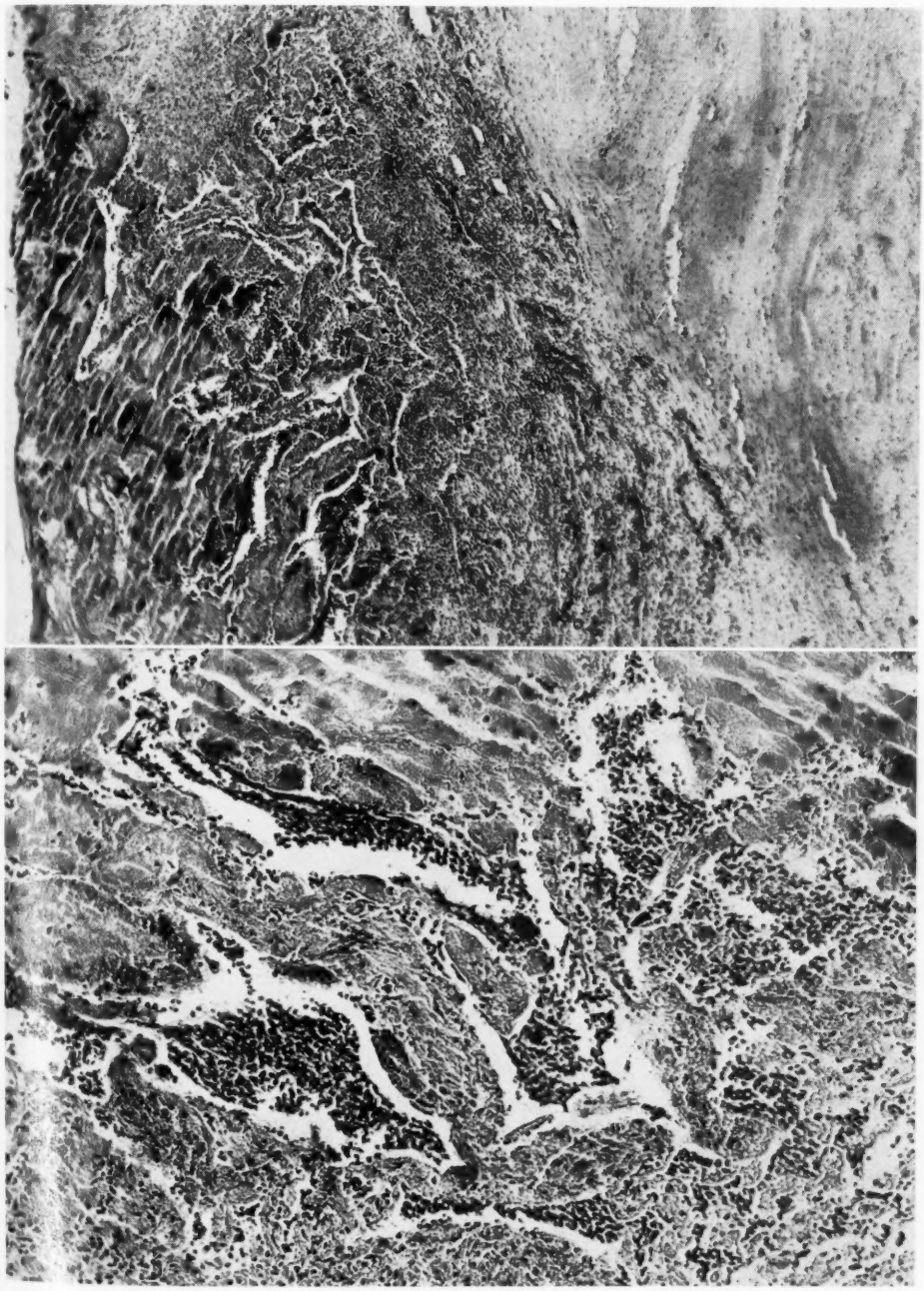

Fig. 7.

Fig. 6.—(Case 1.) Low power photomicrograph of the sessile vegetation designated as area 6 in Fig. 1. Superficially to the left there are fibrin and bacterial colonies. In the right there are many leucocytes and granulation tissue. On the extreme right is the fibrotic valve.

Fig. 7.—(Case 1.) A higher power photomicrograph of the central area of Fig. 6. Note the clots filled with polymorphonuclear leucocytes representing destroyed bacterial colonies.

There is a large partially organized vegetation on the mitral valve (Fig. 12). In the area outlined there is a sharp line of demarcation between the necrotic part and the proliferating fibroblasts. This is taken to mean that the necrotic component of the vegetation organizes slowly or not at all. This conclusion is supported by the better known examples from general pathology of slow organization of the necrotic centra of caseous tubercles and gummas.

CASE 4.—University of Minnesota Autopsy No. 44-999. A 37-year-old white woman was admitted to the hospital on May 5, with a three months' history of chills, fever, and weakness of the left side of her face, the right arm, and the right leg. Despite previous treatment with large amounts of sulfanilamide in another hospital, the blood culture showed *Str. viridans*.

The heart was enlarged to the left, the liver and spleen were palpable, and there was a large umbilical hernia. There were many petechiae on both conjunctivae. Penicillin was started May 6 and given intravenously, 100,000 units every twelve hours. The patient improved, and the blood culture showed no streptococci after twenty-four hours. New petechiae appeared in the conjunctivae, however, and on May 30 the patient suddenly developed sharp abdominal pain localized in the umbilical region. An exploratory laparotomy, performed because of the possibility of a strangulated abdominal hernia, revealed a generalized peritonitis. The patient died on the operating table. Penicillin had been continued to the day of death, the total dose being 4,200,000 units.

The anatomic diagnosis included: chronic endocarditis of the mitral valves; subacute bacterial endocarditis of the mitral valve; suppurated infarcts of the spleen with rupture and peritonitis; focal embolic glomerulonephritis; bilateral pulmonary atelectasis; and acute arteritis of the hepatic artery with thrombosis.

This case is used to illustrate advanced healing of a small vegetation. In Fig. 14 there are three distinct strata: a central calcified core, a middle layer of hyalinized and necrotic tissue, and a superficial layer of loose proliferating fibrous tissue.

The appearance in Fig. 14 could be compared to that in Fig. 2, if the central part of the latter were to become compacted and the centrum to calcify.

Fig. 15, from a case not given in detail (University of Minnesota 44-1468), shows calcification of an individual bacterial colony.

CASE 5.—Barnes Hospital Autopsy No. 11,358. A 46-year-old white man was admitted to the hospital on July 3, complaining of pain and edema in the left leg for one month, exertional dyspnea for three weeks, and a pruritic rash for three days. The patient had an attack of rheumatic fever at 21 years of age and another a year later.

The heart was enlarged and there was a harsh systolic murmur over the precordium. The liver was enlarged. On July 5 a blood culture showed hemolytic streptococci, the patient had a fever, and the spleen was palpable.

On July 15 penicillin was started, 20,000 units every two hours intramuscularly. After July 16, the next day, all blood cultures were negative and the temperature was normal. Penicillin was discontinued August 9, after a total of 6,080,000 units had been given in twenty-six days. The patient was discharged, improved, on August 23.

He was readmitted on August 30, complaining of increased dyspnea and edema of the ankles. On September 20 the patient had a sudden attack of pulmonary edema and died shortly afterward.

The anatomic diagnosis included chronic endocarditis of the aortic valve with calcification and aortic stenosis.

The aortic valve (Fig. 16) is greatly deformed with a thin base and a large bulbous calcified edge. The calcified masses are surrounded by a loose fibroblastic tissue infiltrated with lymphocytes and large mononuclear cells (Fig. 17). In other regions, especially near the surface, there are endothelial-lined spaces (Fig. 18), variable in size and outline but corresponding in general architectural pattern to the lacunae left by destruction of bacteria shown in Fig. 7. The surface is lined by endothelium, and all evidence of a vegetation is absent.

Fig. 8.

Fig. 9.

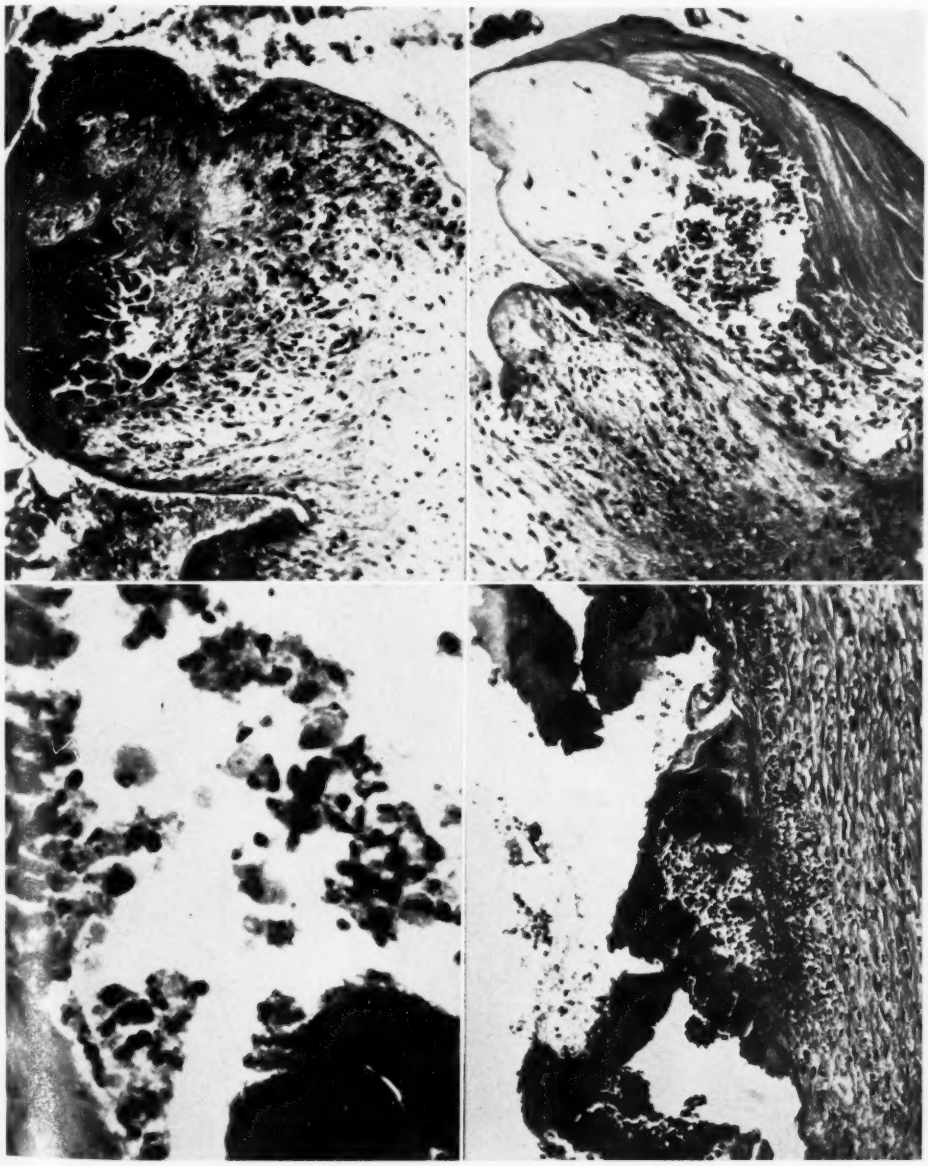

Fig. 10.

Fig. 11.

Fig. 8.—(Case 2.) Partial organization of a bacterial colony.

Fig. 9.—(Case 2.) Clear area in the superficial part of the vegetation partially filled with leucocytes and with the remnants of a bacterial colony.

Fig. 10.—(Case 2.) Active phagocytosis of bacteria about a large colony.

Fig. 11.—(Case 2.) Invasion of the bacterial colonies from below in a mural vegetation. Lesion on the wall of the left auricle.

Fig. 12. Fig. 13.

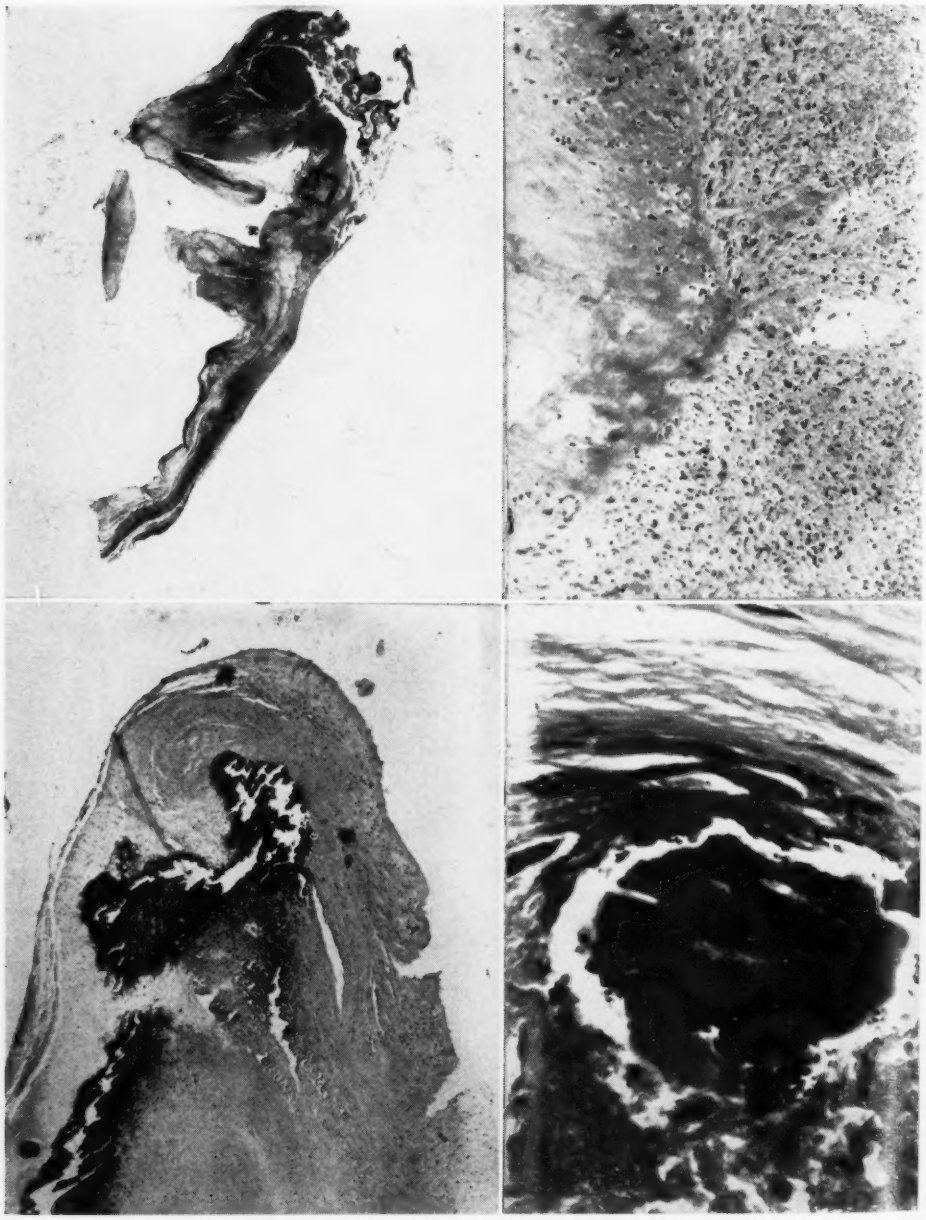

Fig. 14. Fig. 15.

Fig. 12.—(Case 3.) Partially organized vegetation on the mitral valve.

Fig. 13.—(Case 3.) From the designated area on Fig. 12 to show the sharp line of demarcation between the necrotic part of the vegetation and the organizing part.

Fig. 14.—(Case 4.) Calcification of the central part of a completely organized vegetation surrounded by a layer of cartilaginous connective tissue and covered on the surface by a loose fibroblastic tissue.

Fig. 15.—(Case not given in detail.) Calcification of a bacterial colony.

Fig. 16. Fig. 17.

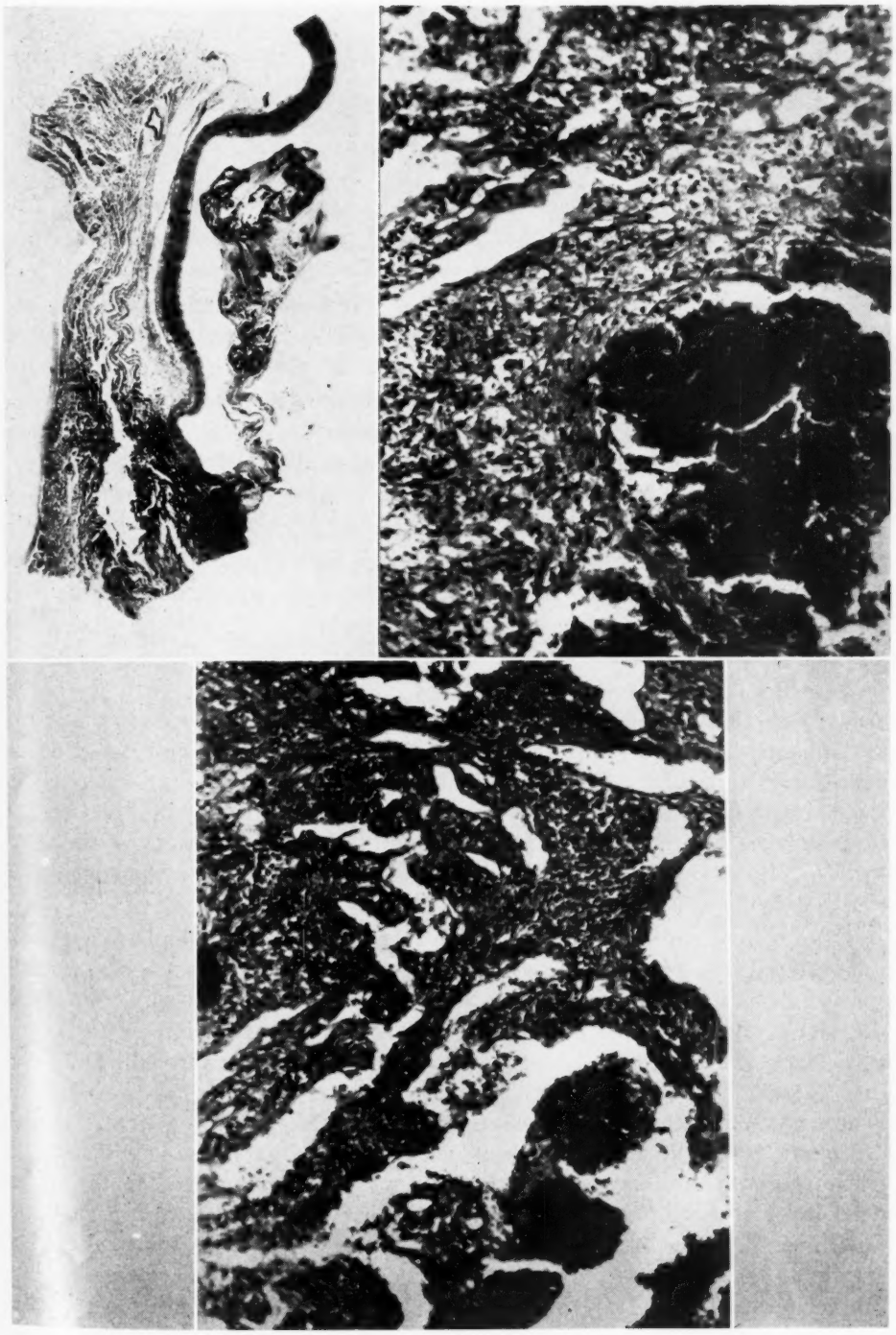

Fig. 18.

Fig. 16.—(Case 5.) Calcified nodules of the aortic valve in a patient who died two months after apparent cure of subacute bacterial endocarditis.

Fig. 17.—(Case 5.) A calcified nodule surrounded by loose connective tissue.

Fig. 18.—(Case 5.) Large endothelial lined spaces in the superficial part of the aortic valve shown in Fig. 16.

The observations of this valve with advanced healing serve to confirm the conclusions reached from study of the earlier stages of healing seen in the other cases. The vegetation organizes or calcifies, and the bacteria are destroyed, the spaces occupied by them becoming endothelized.

HEALING IN SUBACUTE BACTERIAL ENDOCARDITIS

On the basis of these observations, healing in subacute bacterial endocarditis may be arranged as five processes: covering of the exposed surface of the vegetation with fibrous tissue, invasion of the layer of colonies and phagocytosis of bacteria, calcification of bacterial colonies, hyalination and calcification of the central core of the vegetation, and endothelization of the spaces and clefts in the vegetation.

In order to understand the first process, covering of the exposed surface of the vegetation with fibrous tissue, it is necessary to return to the discussion of the nature of the vegetation in bacterial endocarditis. To recapitulate, it is composed of a central core of necrotic tissue, a layer of bacterial colonies embedded in fibrin, and a superficial thin layer of fibrin. The necrotic core is similar to other foci of dead tissue such as the caseous nodule of tuberculosis. There is no network of fibrin on which an invading granulation tissue may form. Hence, it undergoes organization slowly or not at all. On the other hand, the superficial layer is fibrin and is readily and quickly replaced by fibrous tissue. The limiting factor is the state of the fibrous tissue at the edge of the vegetation. If this region is well vascularized and if the fibrous tissue is not too collagenous and hyalinized, proliferation will proceed from some or all directions. In time the central core will be completely covered. Concurrently, the fibrous tissue about the base of the vegetation is active in isolating the necrotic mass and invading it along the few septa and at places where a net work of fibrin exists. The end result is a central necrotic mass completely surrounded by tissue.

The second process, invasion of the layer of colonies and phagocytosis of the bacteria, is a phenomenon seen in all bacterial diseases. In the absence of a specific chemical or biologic agent which will kill bacteria, the only method for disposal of the bacteria is to bring active phagocytes to the infected focus. In subacute bacterial endocarditis of a valve, this is not possible until the first process, covering of the surface with tissue, has taken place. A possible alternate is organization of the necrotic center, but it has been pointed out that this does not occur except in small superficial vegetations. In other words, leucocytes cannot migrate through the necrotic core, nor will they go through the superficial layer of fibrin from the blood in the cavities of the heart in sufficient numbers. That the latter is attempted is suggested by the alinement of leucocytes on the surface of the vegetation. As fibrinoblasts and capillaries replace the peripheral layer of fibrin, the adjacent bacterial colonies are broken apart and the bacteria are phagocytized and destroyed, at least in large part.

For reasons not apparent from the histologic study, some bacterial colonies undergo calcification, the third process. Perhaps the bacteria die and attract calcium salts in the same manner that necrotic tissue does.

The fourth process, hyalination and calcification of the necrotic part of the vegetation, is an undesirable feature of healing. As previously pointed out, necrotic tissue is not ideal soil for organization. There is no pre-existent framework of fibrin for the fibrinoblasts and capillaries. Therefore there is encapsulation with hyalination and calcification. This process does not involve any significant degree of contraction or decrease in size. Hence the end result is a greatly deformed nodular valve, similar to that of calcific stenosis.

As the bacterial colonies are destroyed and as the necrotic core becomes lobulated, clefts and spaces appear in the vegetation. Endothelial cells from the surface or from sprouting capillaries grow into and line the spaces, the fifth process.

In summary, healing in subacute bacterial endocarditis is essentially the conversion of a vegetative endocarditis into a chronic endocarditis characterized by superficial vascularized connective tissue and central nodular calcification. Only time will tell whether or not the latter disease is functionally significant. Reports so far suggest that some patients develop cardiac failure as a result of the calcific stenosis, as did one of the patients reported on in this paper.

TREATMENT OF SUBACUTE BACTERIAL ENDOCARDITIS

Comparison of the observations in those patients treated with penicillin and in those patients who received no specific therapy indicates that there is some evidence of healing in all, regardless of the form of treatment. However, more advanced healing is observed after treatment with penicillin.

Logically there are four possible procedures by which healing might be facilitated: (1) Control the further growth of bacteria; (2) bring phagocytes to the bacteria; (3) promote the growth of granulation tissue; and (4) aid in the final disposal of necrotic tissue.

Clearly, penicillin is effective in the first procedure, control of further growth of bacteria. This inhibition, regardless of how produced, will be reflected in greater phagocytosis and proliferation of tissue, since both are unfavorably influenced by active multiplication of bacteria. The sooner the inflammation is brought to a standstill the more effective the final disposal of the necrotic debris. Hence, penicillin by an action of simple bacteriostasis exerts an effect on all aspects of healing. Therefore, in the sections from patients treated with penicillin, there is more extensive and advanced healing than in those not so treated.

The dose and duration of treatment are largely outside the domain of the pathologic anatomist. However, it is suggested that a blood level effective bacteriostatically on the organism of the patient be maintained for the period it takes for the layers of bacterial colonies and fibrin to be replaced by tissue. The actual range is probably 200,000 to 1,000,000 units per day in not less than six divided doses intramuscularly for three to six months.

This study was originally undertaken to determine whether or not better forms of treatment could be developed. Penicillin has a direct effect on only one of the four possible modes of attack. Perhaps when really effective agents become known, it may be possible to accelerate the phagocytosis.

Some of the factors determining the healing of wounds are already understood. The vegetation is a wound which must be healed in part by fibrosis. Among other things, an adequate supply of protein and vitamin C is necessary for rapid proliferation of normal fibroblasts. The most careful attention should be given to the nutritional status of the patient with subacute bacterial endocarditis, particularly to the level of the plasma albumin and to the adequacy of available vitamin C.

If the vegetation is largely necrotic tissue, it follows that it does not grow by addition of fibrin on the surface. Hence, there is no basis for the use of an anticoagulant such as heparin to prevent a process which does not occur.

Whether or not it is feasible or desirable to accelerate calcification, will be left for others to decide. It has been reported that calcification of caseous tuberculous nodules in rabbits may be hastened by a high calcium diet and vitamin D. Perhaps a similar procedure initiated toward the end of active treatment would be worth while. Possibly further research may reveal a technique for disposal of the necrotic tissue which does not lead to calcification and deformation of the valve.

PATHOGENESIS OF SUBACUTE BACTERIAL ENDOCARDITIS

It is not possible to study the what, that is, the pathologic anatomy of a disease, for long and not wonder about the how and why.

How and why do people develop subacute bacterial endocarditis? Of the thousands who have rheumatic fever, only a small percentage have this complication. Of the thousands who have a tooth extracted or have an acute upper respiratory infection, only a very few develop an endocarditis. Even when these two states, lesions of rheumatic fever and streptococci, are present together, the known incidence cannot account for the onset of subacute bacterial endocarditis.

The basic part of all vegetations is the same, the necrotic core of a valve. This is true for the verrucae of rheumatic fever, the vegetation of nonbacterial thrombotic endocarditis, and for the lesions of bacterial endocarditis. Bacterial endocarditis differs from the others only in that there is a superficial layer of bacteria and fibrin. May it not be postulated, then, that bacterial endocarditis consists of two parts, a nonbacterial primary part and a bacterial secondary part?

It is well known that nonbacterial thrombotic endocarditis is a not uncommon observation at autopsy in those who have died of debilitating diseases. Perhaps it occurs and heals more frequently than is suspected. It might account for the fibrous thickening of valves and adhesions at the commissures of the aortic valves observed in 60 to 90 per cent of all hearts at autopsy.

It is therefore submitted as a working hypothesis that if a person has a nonbacterial endocarditis, either rheumatic or thrombotic, at a time when he also develops a bacteriemia with *Str. viridans,* he or she may develop subacute bacterial endocarditis.

This would explain (1) the known association of lesions of rheumatic fever and subacute bacterial endocarditis in the same heart, (2) the frequency of

lesions of subacute bacterial endocarditis on previously damaged valves, and (3) the low over-all incidence of subacute bacterial endocarditis in relation to previously postulated causal factors.

SUMMARY

A comparison has been made of the lesions of the heart in subacute bacterial endocarditis in twenty-two patients treated with penicillin and in eight patients given no specific therapy.

Penicillin promotes healing in subacute bacterial endocarditis, but the basic processes of healing are not modified. These processes are: covering of the exposed surface of the vegetation with fibrous tissue, invasion of the layer of colonies and phagocytosis of bacteria, calcification of bacterial colonies, hyalination and calcification of the central core of the vegetation, and endothelization of the spaces and clefts in the vegetation.

In some patients, healing is accompanied by excessive calcification, and the result is a calcific stenosis of the valve.

It is postulated that the vegetative lesions of subacute bacterial endocarditis consist of two parts, a nonbacterial (rheumatic or thrombotic) primary part and a bacterial secondary part.

EFFECTIVENESS OF CONJUGATED FORMS OF FOLIC ACID IN THE TREATMENT OF TROPICAL SPRUE

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FOLIC acid (pteroylglutamic acid) occurs naturally not only as the free vitamin, but also in the form of various complexes or conjugates. Two of these complexes, both polyglutamates, have been isolated in pure form, one from yeast and one from a filtrate obtained from an aerobic fermentation of a diphtheroid type organism. The folic acid complex of yeast, often termed vitamin B_c conjugate, was identified by Pfiffner and co-workers[1, 2] as pteroylhexaglutamylglutamic acid. The other naturally occurring complex, usually termed fermentation folic acid or fermentation *Lactobacillus casei* factor, was described by Hutchings, Stokstad, and their collaborators[3, 4] and was shown to be a triglutamate (pteroyldiglutamylglutamic acid).

The ability of these complexes of folic acid to affect the growth of those organisms unable to synthesize folic acid shows certain interesting differences. Thus, fermentation folic acid appears to be utilized effectively by *L. casei*, exerting an effect deviating from that of crystalline pteroylglutamic acid (PGA) only as the molecular weights differ; for *Streptococcus faecalis (Streptococcus lactis R)* the compound has only about one-fiftieth the activity of pteroylglutamic acid. The conjugate of yeast (heptaglutamate) is essentially unable to stimulate the growth of either microorganism. In animal species known to require folic acid (monkey, chick, rat), each of the conjugates exerts an effect essentially equivalent to its folic acid content, and it is probable that this activity results from these compounds serving as a source of free folic acid, split off in each case by the action of a group of enzymes widely distributed in tissues and generally termed conjugases. The possibility has not been excluded, however, that the conjugates may also be utilized as such.

The effectiveness of folic acid in the treatment of pernicious and other forms of macrocytic anemia, including sprue, led to the investigation of these complexes as examples of the types of compounds from which human subjects presumably must derive a considerable portion of their supply of folic acid.

It has already been reported by Welch and associates[5, 6] and by Bethell and co-workers[7] that the conjugated folic acid of yeast is little or not at all utilized by patients with pernicious anemia, although normal human subjects demonstrated their ability to derive folic acid from the same lots of this heptaglutamate by a marked augmentation of the urinary excretion of the free vitamin following the oral or parenteral administration of the conjugate. It was suggested by both groups of workers that the folic acid deficiency char-

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acteristic of pernicious anemia might be due, at least in part, to the inability of such patients to obtain adequate amounts of the free vitamin from the naturally occurring complexes found in the diet and that as a result of this biochemical defect the supply of free folic acid is insufficient for the maintenance of normal hematopoiesis. It was suggested by Welch and associates[5, 6] that liver factors have a more deep-seated action than that solely concerned with correcting a defect in the utilization of conjugates of folic acid, since such extracts have produced a striking reticulocytosis following an inadequate response to prolonged dosage with synthetic folic acid.

Another complication has been offered by the finding that some patients with pernicious anemia, of a type as yet indistinguishable from that previously described, are able to utilize concentrates of the yeast form of conjugated folic acid. Of such a type, Sharp[8] has observed two examples and Castle,[9] one; while one patient with pernicious anemia in remission induced by liver extract, but maintained for more than six months on folic acid without liver extract, has been observed by Heinle and associates to utilize the heptaglutamate, as evidenced by the urinary elimination, in the form of free *L. casei* factor, of a large proportion of the administered conjugate.

It is clear that some patients with pernicious anemia are unable to release folic acid from the preparations of heptaglutamate used,* while in other patients this biochemical defect is not present. Conceivably, such differences in handling the conjugate might reflect etiologic differences in the disease or might represent quantitative differences in the degree of deficiency of intrinsic factor or of erythrocyte maturation factor. On the other hand, it is now known that one or more substances occur naturally (for example, in yeast) that are capable of competitively inhibiting some of the conjugases which release folic acid from pteroylhexaglutamylglutamic acid.† The possibility has not yet been excluded that the concentrates of vitamin B_c conjugate used in patients with pernicious anemia, who are able to derive folic acid from them, differed from those which caused neither a clinical response nor the appearance of folic acid in the urine. Although one lot of material that produced no response, in small daily doses or in a single dose of 30 mg.,[6] represented apparently pure, crystalline pteroylhexaglutamylglutamic acid, other studies were carried out with concentrates that probably contained inhibitors of conjugases.‡ It is not inconceivable that one of the defects in patients with

*Eight patients of this type have been described in reports by investigators at Ann Arbor and Cleveland.

†Personal communication from Dr. John R. Totter, School of Medicine, University of Arkansas, Little Rock, Ark. The inhibitory substance used by Dr. Totter was supplied by Dr. Sarah Ratner and corresponds to the glutamic acid polypeptide of *p*-aminobenzoic acid (Ratner, S., Blanchard, M., and Green, D. E.: Isolation of a Peptide of *p*-Aminobenzoic Acid From Yeast, J. Biol. Chem. **164**: 691, 1946.) It was found that the polypeptide has an affinity from five to ten times as great as vitamin B_c conjugate for preparations of conjugase derived from chicken pancreas and rat liver, but the response was not always obtainable and the inhibitor appeared not to be identical with a material causing inhibition of a conjugase derived from hog kidney. Using a preparation generously supplied by Dr. Ratner, two of us (V. D. W. and E. M. N.) have found that extracts of human bone marrow, whose ability to release folic acid from pteroylhexaglutamylglutamic acid was measured at pH 4.5, are inhibited by the polyglutamate of *p*-aminobenzoic acid, but quantitative data are not yet available.

‡In a personal communication Dr. J. J. Pfiffner has stated that the concentrates we employed were found by Dr. O. D. Bird to be essentially free of all inhibitor for hog kidney conjugase; however, information is not yet available concerning the presence of inhibitors (such as the glutamic acid polypeptide of *p*-aminobenzoic acid) of the conjugase found in human bone marrow.

pernicious anemia will be found to consist of a partial to complete inability to inactivate such inhibitors of conjugases; this possibility has been anticipated in previous papers.[5, 6]

Spies[10] has recently reported that fermentation folic acid, in a daily intramuscular dose of 3 mg., given during a period of eleven days, caused a submaximal reticulocyte response in a patient with pernicious anemia in relapse. Urinary excretion data were not presented. Although it has not been shown that treatment with liver extracts will restore the ability to release folic acid from its conjugates in patients previously unable to utilize such material, it is worth noting that the patient studied by Spies had received injections of liver extracts at some unstated time in 1946. It will be necessary and desirable to test the triglutamate in patients with pernicious anemia in severe relapse who have received no exogenous source of liver factors, at least for several months. However, the most recent findings, some of which are presented here, suggest that fermentation folic acid will not require liver extract factors to permit its utilization.

The supply of pteroyldiglutamylglutamic acid has been exceedingly limited and it has not been possible to obtain extensive information concerning its effect in tropical sprue. Similarly, the supply of the yeast form of conjugate has been restricted by the great difficulties attendant to isolation. In this report are described the striking clinical effect and hematologic responses, together with data on the urinary excretion of folic acid, obtained in two patients with tropical sprue. These patients were treated (by R. M. S. and R. M. S., Jr.) in the School of Tropical Medicine and University Hospital in San Juan, while the analytic studies of the urine were carried out in Cleveland (by A. D. W., R. W. H. and E. M. N.).*

CLINICAL OBSERVATIONS

Patient M. Q. (Unit history A-8757), a white, Puerto Rican man, 48 years of age, was admitted to the University Hospital on June 12, 1946, with the chief complaints of weakness, diarrhea, and weight loss (about forty pounds) during a period of about one year.

He stated that he was feeling well and that he had eaten what he considered to be a well-balanced diet until one year prior to admission, when he first noticed diarrhea. The diarrhea was described as watery, greenish-yellow, foamy, without mucus or blood, with from three to ten stools daily. With the onset of diarrhea, the patient developed flatulence and anorexia. Soon thereafter he noticed soreness of the mouth, with gradually increasing generalized weakness.

A few months after the onset of illness, a few injections of concentrated liver extract were given at irregular intervals and in inadequate doses. He received the last injection of liver extract two days before hospitalization and fourteen days before injections of fermentation folic acid were started.

On physical examination the patient was found to be in a cachectic condition, extremely pale, and obviously very weak and malnourished. A superficial, dry, scaly, brownish dermatosis was noticed over both ankles and wrists. The tongue was pale and fissured, and, although covered by papillae, these were very small and some of them over the tip and margins of the tongue were definitely inflamed. No aphthae were seen.

On admission, examination of the blood disclosed 1,200,000 erythrocytes per cubic millimeter; hemoglobin, 5.8 Gm. per 100 c.c.; leucocytes, 5,400 per cubic millimeter; mean cell

*The materials used in the studies were obtained through the courtesy of the Lederle Laboratories, Inc., Pearl River, N. Y. (fermentation folic acid) and of the Laboratories of Parke, Davis & Co., Detroit, Mich. (concentrate of vitamin B_c conjugate).

volume, 160 $c\mu$; the corrected sedimentation rate was 18 mm. per hour. Bone marrow aspiration revealed 25 per cent megaloblasts and 12 per cent normoblasts, with an erythrocytic series of 55 per cent and a granulocytic series of 37.8 per cent. Chemical findings in the blood were within normal limits, except for a serum protein level of 4 Gm. per 100 c.c., with a normal albumin-globulin ratio. Gastric analysis showed hypochlorhydria. Serologic and urinary findings were negative. Examination of the stool showed free fatty acids, soapy fats, and neutral fat (videinfra).

Laboratory Examinations.—Urine analysis revealed no abnormalities. Examination of stools disclosed hookworm ova. Analytic findings in blood were as follows: nonprotein nitrogen, 26.4 mg. per 100 c.c.; urea nitrogen, 12.7 mg. per 100 c.c.; sugar, 72.7 mg. per 100 c.c.; cholesterol, 108.6 mg. per 100 c.c.; phosphorus (inorganic), 2.7 mg. per 100 c.c.; calcium, 7.8 mg. per 100 c.c.; phosphatase, 4.7 Bodansky units; van den Bergh reaction, delayed; total serum protein, 4.0 Gm. per 100 c.c. (albumin, 2.6 Gm. per 100 c.c.; globulin, 1.4 Gm. per 100 c.c.); icterus index, 3.2 per cent; chlorides (as NaCl), 461.0 mg. per 100 c.c.

Gastric analysis after stimulation with histamine was as follows:

	FREE HYDROCHLORIC ACID (°)
Fasting	0
First	40
Second	26
Third	12
Fourth	0
Lactic acid, absent	

Biophotometric curve began at 26 and finished at 63 (normal, 55 to 100). Analysis of the dry solids (22 per cent) of a stool sample was as follows: total fat, 78.7 Gm. per 100 Gm.; free fatty acids, 65.7 Gm. per 100 Gm.; soaps, 0.9 Gm. per 100 Gm.; total split, 66.7 Gm. per 100 Gm.; neutral, 9.4 Gm. per 100 Gm.; unsaponified, 2.6 Gm. per 100 Gm.

Treatment.—The patient was placed on what we have referred to as a preliminary sprue diet, and this dietary regime, high in carbohydrates and very low in animal proteins, was maintained throughout the experiment. Twelve days after admission, when all laboratory examinations had been performed and various base lines established, daily intramuscular injections of fermentation folic acid were begun. The solution used was prepared by one of us (A. D. W.), in San Juan from a powder of 65 per cent purity. This material was dissolved in water and the pH adjusted to approximately 7.4 with the aid of sodium hydroxide. The solution was designed to contain, in each 2 c.c., approximately 3.0 mg. of potential folic acid. After autoclaving at 15 pounds pressure for fifteen minutes, a sample of the solution was withdrawn aseptically, hermetically sealed in a sterile capillary tube, and taken to Cleveland for analysis. Microbiologic assay (*L. casei* and *Str. faecalis*) before and after treatment with hog kidney conjugase[11] indicated that each daily dose (2 c.c.) of the solution contained approximately 4.9 mg. of fermentation folic acid, equivalent to about 3.1 mg. of pteroylglutamic acid. When treatment with fermentation folic acid was initiated, on June 24, 1946, the following hematologic observations were made: erythrocytes, 800,000 per cubic millimeter; hemoglobin, 4.2 Gm. per 100 c.c.; mean cell volume, 175 $c\mu$; leucocytes, 2,300 per cubic millimeter; the reticulocytes, which originally were at an 8 per cent level, now composed 2 per cent of the circulating red cells. The patient was having from six to eight bowel movements daily and the body weight was only 96 pounds.

Three days after initiation of treatment with fermentation folic acid, the patient volunteered the statement that he felt better; the bowel movements had decreased to three daily; the number of megaloblasts in the sternal bone marrow was reduced to 1.2 per cent (from 25 per cent); the appetite had improved markedly and the patient experienced a sense of well-being. In spite of the inadequate diet, there occurred a definite improvement in the number and character of the stools. By the fourth day the stools were of normal consistency.

TABLE I. HEMATOLOGIC DATA OBTAINED IN PATIENT WITH SPRUE*

DATE	HEMOGLOBIN (GRAMS PER 100 C.C.)	ERYTHROCYTES (MILLIONS PER C.MM.)	LEUCOCYTES (THOUSANDS PER C.MM.)	RETICULOCYTES (PER CENT)
6/24/46	4.2	0.80	2.3	2.0
6/26/46	4.8	0.82	1.7	5.2
6/27/46	4.8	0.81	1.1	6.0
6/28/46	4.0	0.80	1.0	18.0
7/ 1/46	5.4	1.21	2.1	12.4
7/ 2/46	6.6	1.20	3.6	17.8
7/ 3/46	5.8	1.28	3.7	12.8
7/ 4/46	7.2	1.59	3.5	15.8
7/ 5/46	6.8	1.75	2.2	11.0
7/ 6/46	--	--	--	11.4
7/ 8/46	7.2	1.73	4.3	9.6
7/ 9/46	7.6	1.83	3.8	9.4
7/10/46	8.1	2.09	4.9	6.8
7/12/46	8.4	2.39	5.6	11.2
7/16/46	10.5	2.60	3.0	11.4
7/24/46	10.1	3.03	5.3	3.2
7/29/46	11.4	3.46	4.7	3.8
8/ 5/46	12.8	3.52	4.0	0.3
8/12/46	12.0	3.66	5.1	0.3

*The patient (M. Q.) was given daily intramuscular injections of 4.9 mg. of fermentation folic acid (equivalent to 3.1 mg. of free folic acid), beginning June 24 and continuing through July 7, 1946 (14 injections). Additional therapy with liver extract (1 c.c. daily, containing 15 units) was begun on July 4 and was continued through Aug. 4, 1946.

On the fourth day of treatment the reticulocyte level had reached a peak of 18 per cent, and a level of about 18 per cent was maintained until the tenth day of treatment, July 4, when the daily administration of 1 c.c. of liver extract (15 units) was begun, in addition to the same dose of fermentation folic acid. On this day the erythrocyte count was 1,590,000 per cubic millimeter of blood and the hemoglobin level was 7.2 Gm. per 100 cubic centimeters. The number of leucocytes and of platelets had shown little change. The body weight had increased by six pounds.

Following the injections of liver extract, in addition to those of fermentation folic acid, the number of white blood cells and platelets increased, and there was a persistence of the reticulocytosis, a progressive increase in the number of erythrocytes and in the amount of hemoglobin, and a definite change toward complete recovery.

On July 16, twenty-two days after treatment was begun, the erythrocyte count was 2,600,000 per cubic millimeter; the hemoglobin level, 10.5 Gm. per 100 c.c.; mean cell volume, 141 $c\mu$; leucocytes, 3,000 per cubic millimeter; and the reticulocyte level was 11.4 per cent. The body weight was 112 pounds (an increase of sixteen pounds).

Patient J. Q. (Unit history A-9216), a white man, 53 years of age, was admitted to the University Hospital on Sept. 5, 1946, with the chief complaint of generalized weakness during the past three months, accompanied by foamy diarrhea, a burning sensation of the tongue, flatulence, anorexia, and marked loss of weight. The stools were described as yellowish, abundant, fetid, either liquid or semisolid, with foam and mucus but no blood, and as occurring anywhere from twelve to eighteen times in twenty-four hours.

His past medical history was irrelevant, except for the fact that he had been on a poorly balanced diet for a number of years.

Physical examination revealed a short, poorly nourished, pale, cachectic, white man, who weighed only 68 pounds. The tongue was red and smooth, showing the typical appearance of an atrophic glossitis. No aphtha was found. The rest of the examination, including the neurologic, yielded essentially normal findings.

Roentgenologic study of the small intestine showed segmentation of the jejunum.

Laboratory Examinations.—The stools contained the ova of hookworm. The glucose tolerance curve was flat and showed an increase of only 15 mg. per 100 c.c. Analytic findings in blood were as follows: nonprotein nitrogen, 23.3 mg. per 100 c.c.; urea nitrogen,

13 mg. per 100 c.c.; cholesterol, 120 mg. per 100 c.c.; phosphorus (inorganic), 4 mg. per 100 c.c.; calcium, 8.6 mg. per 100 c.c.; phosphatase, 1.7 Bodansky units; van den Bergh reaction, delayed; total serum protein, 5.8 Gm. per 100 c.c. (albumin, 3.2 Gm. per 100 c.c.; globulin, 2.6 Gm. per 100 c.c.); icterus index, 9.2 per cent.

Gastric analysis after stimulation with histamine was as follows:

	FREE HYDROCHLORIC ACID (°)
Fasting	0
First	0
Second	60
Third	76
Fourth	44

Wassermann and Kahn tests were negative. Hanger cephalin flocculation test was 3 plus. No urobilinogen was found in the urine.

Analysis of the dry solids (15.5 per cent) of a stool sample was as follows: total fat, 58 Gm. per 100 Gm.; free fatty acids, 43.5 Gm. per 100 Gm.; soaps, 1.9 Gm. per 100 Gm.; total split, 45.4 Gm. per 100 Gm.; neutral, 9.3 Gm. per 100 Gm.; unsaponified, 3.2 Gm. per 100 Gm.

Treatment.—This patient had never received liver extracts, yeast, or vitamin supplementation of his diet. He was placed on the preliminary sprue diet, high in carbohydrates and very low in animal proteins, and maintained on this diet throughout the experiment. On Sept. 16, 1946, when oral therapy with a concentrate of vitamin B_c conjugate was initiated, the weight was 73 pounds and the hematologic findings were as follows: erythrocytes, 1,320,000 per cubic millimeter; hemoglobin, 7.2 Gm. per 100 c.c.; leucocytes, 1,400 per cubic millimeter; mean cell volume, 159 $c\mu$. No reticulocytes were observed. The sternal marrow showed 17.8 per cent megaloblasts.

The concentrate of vitamin B_c conjugate used in therapy was supplied by Dr. J. J. Pfiffner and had a labeled potency of 1.2 mg. of potential folic acid per cubic centimeter. Of the total solids, approximately 10 per cent was vitamin B_c conjugate, and less than 0.01 per cent was free folic acid. The concentrate was given orally in daily doses of 7 c.c., containing conjugated folic acid in an amount equivalent to about 8.4 mg. of free folic acid (23.2 mg. as pteroylhexaglutamylglutamic acid).

TABLE II. HEMATOLOGIC DATA OBTAINED IN PATIENT WITH SPRUE*

DATE	HEMOGLOBIN (GRAMS PER 100 C.C.)	ERYTHROCYTES (MILLIONS PER C.MM.)	LEUCOCYTES (THOUSANDS PER C.MM.)	RETICULOCYTES (PER CENT)
9/16/46	7.2	1.32	1.4	0.0
9/17/46	8.7	1.06	2.1	0.0
9/18/46	6.0	1.21	2.4	0.0
9/19/46	5.2	1.13	1.0	0.0
9/20/46	4.8	1.07	0.9	1.4
9/21/46	--	--	--	3.4
9/22/46	--	--	--	14.0
9/23/46	5.8	1.27	3.0	23.8
9/24/46	5.8	1.31	1.7	28.4
9/25/46	5.0	1.56	1.8	33.0
9/26/46	6.0	1.75	2.4	30.4
9/27/46	6.4	1.86	3.3	24.0
9/28/46	7.2	2.14	4.7	23.8
9/30/46	6.8	1.95	2.2	23.6
10/ 1/46	6.4	2.15	3.1	20.0
10/ 2/46	6.6	2.02	3.5	15.2
10/ 8/46	6.4	1.89	6.0	9.4
10/14/46	7.8	2.05	6.0	7.4

*The patient (J. Q.) was given daily oral doses of a concentrate of vitamin B_c conjugate (equivalent to 8.4 mg. of folic acid), beginning September 16 and continuing through Sept. 30, 1946 (15 doses). Additional therapy with liver extract (1 c.c. daily, containing 15 units) was begun on September 26 and was continued through Oct. 3, 1946. Folic acid was begun on October 3 at a level of 10 mg. daily for one week and 2.5 mg. daily thereafter.

Reticulocytes began to appear in the peripheral blood on the fourth day of treatment (1.4 per cent); the following levels were encountered subsequently: 3.4 per cent (fifth day), 14 per cent (sixth day), 23.8 per cent (seventh day), 28.4 per cent (eighth day). A reticulocyte peak of 33 per cent was encountered on the ninth day of treatment. On the tenth day daily subcutaneous injections of 1 c.c. of purified liver extract (15 units per cubic centimeter) were begun. At this time the weight was 73 pounds and the hematologic data were as follows: erythrocytes, 1,760,000 per cubic millimeter; hemoglobin, 6.0 Gm. per 100 c.c.; leucocytes, 2,400 per cubic millimeter; reticulocytes, 30.4 per cent; and mean cell volume, 142 $c\mu$. The subjective and clinical improvement was even more evident in this patient than in the one who received the smaller doses of pteroyltriglutamate.

Four days after the institution of treatment, the patient felt better, his appetite had improved, the tongue showed newly formed papillae, and the stools had diminished in number and had improved in consistency. At this time the megaloblasts in the sternal marrow were only 0.6 per cent. On the eighth day of treatment the stools became formed, and since then there have been only one or two bowel movements daily.

URINARY FINDINGS

Studies of the urinary elimination of folic acid by normal human subjects, and by those with various pathologic conditions, have shown that the daily excretion of the vitamin rarely exceeds 5 μg and usually amounts to from 2 to 4 μg, when the intake is restricted to ordinary dietary sources of the vitamin (and to the intestinal synthesis that presumably occurs). Oral or parenteral administration of synthetic folic acid leads to a prompt augmentation

TABLE III. FOLIC ACID EXCRETION IN URINE OF PATIENTS WITH TROPICAL SPRUE

PATIENT M. Q. (FERMENTATION FOLIC ACID)				PATIENT J. Q. (CONJUGATED FOLIC ACID OF YEAST)			
DATE	URINE VOLUME (C.C.)	L. CASEI ASSAY (μG./C.C.)	OUTPUT OF FOLIC ACID (MG./DAY)	DATE	URINE VOLUME (C.C.)	L. CASEI ASSAY (μG./C.C.)	OUTPUT OF FOLIC ACID (MG./DAY)
6/19-20	1,760	--	<0.0002	9/15-16	990	0.0008	0.0008
6/20-21	1,180	--	<0.0002	9/16-17	--	--	--
6/21-22	2,210	--	<0.0002	9/17-18	850	0.0038	0.0032
6/22-23	890	--	<0.0002	9/18-19	880	0.0015	0.0013
6/23-24	1,560	--	<0.0002	9/19-20	630	0.013	0.008
6/24-25	1,330	0.31	0.41	9/20-21	380	0.180	0.068
6/25-26	650	0.85	0.55	9/21-22	910	0.051	0.046
6/26-27	1,060	0.42	0.44	9/22-23	1,140	0.031	0.035
6/27-28	1,620	1.06	1.71	9/23-24	1,280	0.042	0.054
6/28-29	1,150	0.80	0.92	9/24-25	1,020	0.147	0.150
6/29-30	2,440	1.50	3.66	9/25-26	1,690	0.189	0.32
6/30-7/1	2,920	0.44	1.28	9/26-27	1,280	0.30	0.38
7/ 1-2	2,820	0.60	1.69	9/27-28	1,850	0.50	0.93
7/ 2-3	2,420	0.61	1.47	9/28-29	1,570	0.95	1.50
7/ 3-4	3,270	0.41	1.34	9/29-30	1,720	0.66	1.14
7/ 4-5	1,460	4.90	7.15	9/30-10/1	1,510	0.48	0.73
7/ 5-6	2,460	2.96	7.28				
7/ 6-7	2,140	0.84	1.79				
7/ 7-8	2,000	0.41	0.82				
7/ 8-9	2,520	0.0008	0.002				

Patient M. Q. was given intramuscular injections of fermentation folic acid, equivalent to 3.1 mg. of free folic acid, beginning June 24 and continuing through July 7, 1946 (14 injections). In addition, injections of liver extract (1 c.c. daily, containing 15 units) were begun on July 4.

Patient J. Q. was given daily oral doses of the conjugated folic acid of yeast, equivalent to 8.4 mg. of free folic acid, beginning September 16 and continuing through Sept. 30, 1946 (15 doses). In addition, injections of liver extract (1 c.c. daily, containing 15 units) were begun on September 26.

Additional essays with *Str. faecalis* were carried out on all samples of urine obtained from one patient (M. Q.) and on several samples of urine from the other patient (J. Q.). The assay values found were in all cases in close agreement with those obtained with *L. casei*, a circumstance which indicates that the microbiologically active material in the urine of both patients was free pteroylglutamic acid.

of the amount of the vitamin in the urine and, depending on the size of the dose, to an elimination within twenty-four hours of from 15 to 75 per cent of the administered material. A normal subject given vitamin B_c conjugate intramuscularly, in an amount equivalent to 1 mg. of free folic acid, on each of two successive days, excreted 8.3 per cent in the urine as the free vitamin; the same individual excreted 16 per cent when daily doses of 1 mg. of synthetic folic acid were given intramuscularly. On a dosage regime of about 10 mg. daily, either orally or parenterally, the excretion of the free vitamin in the urine usually ranges between 35 and 50 per cent.

In Table III data are presented on the urinary excretion of free folic acid by the patients who were given fermentation folic acid and the conjugated folic acid of yeast, respectively. Although only those data obtained with *L. casei* are presented in the table, duplicate analyses were run with *Str. faecalis (Str. lactis R)* on all urine samples obtained from the patient given fermentation folic acid and on occasional urine samples from the patient given the heptaglutamate. The very close agreement obtained through the use of the two organisms made it clear that the microbiologically active material present in the urine of both patients was pteroylglutamic acid. Treatment of the urine with preparations of conjugase gave no evidence of the presence in the urine of any conjugated forms of folic acid. The conjugases used exerted their expected activity when their effect on added conjugate was tested in the presence of urine from the patients studied.

DISCUSSION

It is of interest to comment on the remarkably small amount of folic acid excreted, prior to therapy, in the urine of the patients on the preliminary sprue diet. In one of these patients (M. Q.) the amount of folic acid excreted was below that which could be determined by the microbiologic method employed; in the other patient (J. Q.) the amount of vitamin excreted (0.8 μg.) on one day was considerably smaller than is usually encountered in the urine of human subjects. A level of excretion of folic acid, below that easily determined, has also been observed in another patient with sprue on the same diet. It is not claimed that these unusually small amounts of urinary folic acid are necessarily characteristic of sprue since they may reflect the very small amount of the vitamin in the diet, but this diet is characteristic of that employed by those among whom sprue is found. Additional studies of the intake and excretion of folic acid of individuals in areas where sprue is encountered are greatly needed.

The data presented in Table III clearly indicate that pteroyldiglutamylglutamic acid served as a source of free folic acid in the patient with sprue (M. Q.) treated with this conjugate. However, the urinary excretion of the free vitamin was appreciably less during the first three days of therapy than on any of the subsequent days when the triglutamate was administered, a circumstance which suggests that retention was more marked when the need was greatest.

Particularly striking, however, is the relatively enormous excretion of folic acid that occurred during the first days of therapy with liver extract.

It should be pointed out that the daily dose (1 c.c.) of liver extract employed contained less than 0.001 mg. of microbiologically determinable folic acid, even after treatment with enzymes known to release the vitamin from its polyglutamates. Also, several investigators have shown that purified liver extracts comparable to this material exert no favorable effect in chicks, rats, or monkeys made deficient in folic acid. In view of the results previously mentioned, indicating that many patients with pernicious anemia are unable to utilize the conjugated folic acid of yeast, it seems possible that a factor of the liver extracts, presumably the factor concerned with the maturation of erythrocytes, markedly affected the metabolism of fermentation folic acid or of tissue complexes formed as a result of its administration. Since the patient was given daily doses equivalent to only 3.1 mg. of free folic acid, an excretion of over 7 mg. on each of two successive days suggests that stores of conjugated material were broken down to free folic acid much more rapidly than was the case prior to therapy with liver extract, although other explanations must be considered. This patient previously had received intermittent injections of liver extract, the last dose having been given only fourteen days prior to the first dose of fermentation folic acid; further, the presence of hydrochloric acid in the stomach is proof that achylia gastrica did not exist. The presence of a macrocytic anemia that responded to therapy demonstrates that during the pre-treatment period the patient failed either to absorb or to synthesize adequate amounts of the erythrocyte maturation factor. These data strongly suggest that the metabolism of this conjugate of folic acid in sprue is altered significantly by the administration of large doses of a liver extract high in antipernicious anemia activity and itself effective in the treatment of sprue. Since this patient had recently been given injections of liver extracts, it will be necessary to study a hitherto untreated patient in order to demonstrate beyond question that fermentation folic acid can be utilized in sprue without the participation of exogenous factors of liver extract. It will be necessary also to show that therapy with liver extract, after a preliminary period on synthetic folic acid alone, does not augment the urinary excretion of folic acid. Preliminary data obtained in patients with pernicious anemia do not indicate that liver extracts have such an effect.

It is not easy to interpret the findings in the patient with sprue (J. Q.) given pteroylhexaglutamylglutamic acid. However, one observation of considerable significance may be made, namely, that a striking clinical response was obtained with this conjugate, without urinary evidence for the cleavage of other than small amounts of the polyglutamate into free folic acid. The degree of reticulocytosis observed at the peak was maximal for the initial erythrocyte count of 1,320,000 per cubic millimeter of blood. It will be noted that on the seventh day, when the reticulocytes had already risen to about 24 per cent, the urinary excretion of folic acid was only about 54 micrograms. Whether these findings indicate that the conjugate was being utilized as such, or that the folic acid released from the conjugate was unusually well retained, cannot now be decided. That the components of the concentrate were poorly absorbed from the gastrointestinal tract is possible; however, the prompt and strik-

ing improvement in the clinical and hematologic condition of the patient, resulting from oral therapy with the conjugate, indicates that absorption was probably quite complete.

Following the parenteral administration of liver extract, in addition to oral dosage with the concentrate of heptaglutamate, urinary excretion of folic acid increased markedly, although not as promptly or as strikingly as in the patient given fermentation folic acid. In fact, from the data available, it is not possible safely to attribute the marked increase in folic acid excretion during the last four days of the experiment, entirely to the effect of the liver extract on the metabolism of the conjugate. The progressive increase in the daily excretion of free folic acid, which was observed prior to the administration of liver extract, suggests the possibility that the later effect might have occurred in the absence of the liver factor. In the light of other observations, however, it appears more likely that a factor of highly purified liver extract, presumably the erythrocyte maturation factor, influenced the ability of this patient with sprue to release folic acid from the conjugate of yeast or from complexes stored in the tissues. If such an assumption is justified, it follows that one of the biochemical defects resulting from a deficiency of the erythrocyte maturation factor may be an abnormality in the derivation of folic acid from its conjugates; it is possible that this abnormality may lie in the mechanism through which the body copes with inhibitors of the enzymes responsible for the release of the vitamin from its naturally occurring complexes. A defect of this type could be either partial or complete, possibly reflecting the fact that variable degrees of deficiency of intrinsic factor and probably of erythrocyte maturation factor are encountered.

SUMMARY

Following the parenteral administration of fermentation folic acid (pteroyldiglutamylglutamic acid) in a dose equivalent to 3.1 mg. of pteroylglutamic (folic) acid daily, the clinical and hematologic manifestations of sprue were rapidly and strikingly improved in a patient previously treated with liver extracts. Urinary studies showed that the patient excreted, prior to treatment, amounts of the free vitamin significantly lower than those usually encountered in normal subjects or those with pernicious anemia. Administration of the conjugate caused the elimination in the urine of free folic acid in an amount indicating that a very large proportion of the conjugate was converted in vivo to the free vitamin, although the amounts excreted during the first three days of therapy suggested that greater retention occurred when the need was greatest. When purified liver extract free of folic acid was administered, in addition to fermentation folic acid, a remarkable increase in the urinary excretion of free folic acid occurred, a level of approximately 7 mg. daily was maintained for two days, a circumstance which suggests that the erythrocyte maturation factor may be concerned with the release of stored complexes of folic acid or that in some other manner it affects the metabolism of folic acid.

Oral administration of a concentrate of vitamin B_c conjugate (pteroylhexaglutamylglutamic acid) of yeast, in daily doses equivalent to 8.4 mg. of free pteroylglutamic (folic) acid, caused rapid clinical improvement in a patient with sprue given no previous treatment with liver extracts, yeast, or vitamins. The reticulocyte response was maximal for a patient with an initial erythrocyte count of 1,320,000 per cubic millimeter. Urinary studies showed at first only small increases in the excretion of the free vitamin, although the amount gradually increased to 0.32 mg. on the ninth day following initiation of therapy. Whether the small amounts excreted are indicative of utilization of the conjugate as such, or whether they suggest an unusually marked retention of free folic acid released from the conjugate, cannot be determined at present. Poor absorption is unlikely in view of the prompt and striking clinical and hematologic improvement observed. Following the administration of purified liver extract (free of folic acid), in addition to the conjugated vitamin, the daily urinary elimination of folic acid was increased to 0.93, 1.50, and 1.14 mg., respectively, on the second, third, and fourth days of additional therapy with the liver extract. Although these findings do not offer conclusive evidence, they strongly indicate that augmentation of the urinary elimination of free folic acid resulted from an effect of a component of purified extracts of liver on the metabolism of this naturally occurring complex of folic acid or on tissue complexes stored as a result of therapy with the conjugate.

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SULFACARBOXYTHIAZOLE: ABSORPTION, EXCRETION, TOXICITY, AND THERAPEUTIC RESULTS IN BACILLARY DYSENTERY AND NONSPECIFIC DIARRHEA

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THE sulfonamides have been classified as absorbable or poorly absorbable, depending upon whether or not they pass through the gastrointestinal wall following oral administration. Poorly absorbable sulfonamides may be superior to those which are more readily absorbed in the treatment of enteric infections caused by sulfonamide-sensitive bacteria and for the preparation of patients who are to undergo bowel surgery for two reasons: first, the poorly absorbable sulfonamides are present in high concentrations within the intestines and, second, most of the systemic toxic reactions are avoided when the poorly absorbable compounds are used.

Sulfacarboxythiazole (2-sulfanilamido-5-carboxysulfathiazole)* is a recently synthesized compound in which a carboxy group is substituted on the thiazole ring of sulfathiazole. It differs from previously synthesized poorly absorbable sulfonamides such as succinylsulfathiazole and phthalysulfathiazole in that these latter drugs are made by the substitution of either a succinyl or phthalyl radical on the para-amino group. The activity of these drugs is dependent on their breakdown in the bowel to sulfathiazole, this being accomplished in the case of sulfacarboxythiazole by decarboxylation and in the case of the other two compounds by hydrolysis. When determinations for sulfacarboxythiazole are made on blood, feces, and urine, the designation "free drug" denotes the amount of sulfathiazole and sulfacarboxythiazole present. The designation "total drug" indicates the quantity of free drug plus the amount of the conjugated or bound form. The latter form of the drug consists of acetylsulfathiazole and acetylsulfacarboxythiazole.

Poth and Ross[1] have shown that phthalysulfathiazole in one-half the dosage was as effective as succinylsulfathiazole in the alteration of the coliform flora of the bowel of man. In equivalent doses the two drugs gave similar low blood concentrations (1 to 1.5 mg. per 100 c.c.) with 10 per cent of the succinylsulfathiazole recovered in the urine, while only 5 per cent of the phthalylsulfathiazole was similarly recovered. White and associates[2] found sulfacarboxythiazole to be similar to phthalysulfathiazole with respect to solubility, absorption, and in vitro bacteriostatic action against dysentery bacilli. Other studies[2-4] have shown sulfacarboxythiazole to be poorly absorbable, equally as bacterio-

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static as sulfaguanidine in vitro and in vivo, as well as nontoxic in man and animals. These preliminary data warranted a clinical study of this drug, the results of which we are reporting in this paper.

ABSORPTION AND EXCRETION

Sixty subjects were given sulfacarboxythiazole in doses varying from 0.1 to 0.5 Gm. per kilogram of body weight per day for periods of three to fifteen days. The total daily dose was calculated for each subject. One-half of this amount was given initially, followed by one-sixth at four-hour intervals. The free and total blood sulfonamide concentrations were determined daily by the method of Bratton and Marshall.[5] Blood was taken for this purpose twenty-five to twenty-seven hours after the initial dose of sulfacarboxythiazole had been administered and at twenty-four hour intervals thereafter. Blood was obtained from two of the subjects four hours after sulfacarboxythiazole had been administered, and the drug was found to be present at that time.

Since the free and total concentrations were essentially the same in over 90 per cent of the determinations, only the total values are recorded. Among the few instances in which the conjugated form was present in significant amounts, it was never found to comprise more than 19 per cent of the total amount of the drug present. In Table I are listed the range of the total blood concentrations found for each dosage schedule. Ninety per cent of the blood concentrations were less than 1 mg. per 100 c.c., although concentrations as high as 2.7 and 4.3 mg. per 100 c.c. were observed in isolated instances. There was no relationship between the duration of treatment and the height of the blood concentrations. In several subjects the highest concentrations were found after two or three days of treatment, whereas lower concentrations occurred later in the course of the study. In general, higher blood concentrations were found in the group of subjects who received the larger doses. Traces of the drug were found in the blood for one to three days after the drug had been discontinued.

Four subjects, two of whom had bacillary dysentery and two of whom were healthy individuals, were given sulfacarboxythiazole for 40 to 112 hours. The total daily dose was calculated on the basis of 0.25 Gm. per kilogram of body weight. This was divided into six equal doses which were given at four-hour intervals. The initial dose was one-half the total daily dose. No restrictions were placed on the intake of food and fluid on any of the subjects, except that the patients with dysentery were maintained on a liquid diet for the first twenty-four hours. Before the drug was administered each subject evacuated

TABLE I. BLOOD CONCENTRATIONS FOLLOWING ADMINISTRATION OF VARIOUS DOSAGES OF SULFACARBOXYTHIAZOLE

DOSAGES (GM./KG. OF BODY WEIGHT)	BLOOD LEVELS (MG./100 C.C.)	NUMBER OF DETERMINATIONS
0.10	0.00 to 1.2	88
0.20	0.45 to 1.0	7
0.25	0.16 to 4.3	8
0.50	0.27 to 2.7	2

TABLE II. AMOUNTS OF SULFACARBOXYTHIAZOLE EXCRETED IN FECES AND URINES OF HUMAN SUBJECTS FOLLOWING ORAL ADMINISTRATION

Part I

SUBJECT	DAY OF STUDY	GRAMS ADMINISTERED	GRAMS RECOVERED			
			FECES		URINE	
			FREE	TOTAL	FREE	TOTAL
H. D.	1	18	15.0	15.0	0.65	0.72
	2	9	9.0	9.0	0.36	0.46
	3		0.72	0.72	0.10	0.13
	4				0.16	0.26
	5				0.02	0.28
B. O.	1	15	9.4	10.0	0.50	0.60
	2	15	14.4	15.0	0.65	0.81
	3	15	10.9	10.9	0.60	0.69
	4	5	5.5	5.7	0.30	0.39
	5		0.078	0.078	0.045	0.57
W. D.	1	12	0.89	0.89	0.21	0.227
	2	18	13.5	13.9	0.35	0.44
	3	18	11.2	11.3	0.35	0.40
	4	18	16.7	16.8	0.34	0.40
	5	12	12.1	12.1	0.28	0.35
	6		12.6	12.61	0.16	0.21
	7		0.013	0.018	0.03	0.035
	8				0.013	0.015
G. L.	1	22	No stool	No stool	0.64	0.67
	2	24	22.0	22.25	1.1	1.4
	3		20.0	20.0	0.4	0.7
	4		0.1	0.1	0.3	0.4
	5				0.1	0.1

Part II

SUBJECT	H. D.	B. O.	W. D.	G. L.
A. Recovered from feces				
Grams free	24.72	40.278	66.563	42.10
Grams total	24.72	41.678	67.168	42.35
Per cent of administered drug	91.55	83.35	86.11	92.06
Per cent conjugated	0	3.4	0.9	0.5
B. Recovered from urine				
Grams free	1.290	2.095	1.733	2.54
Grams total	1.598	2.547	2.077	3.27
Per cent of administered drug	5.92	5.10	2.66	7.11
Per cent conjugated	19.5	17.7	16.5	22.3
C. Recovered from feces and urine				
Grams free	26.010	42.373	68.296	44.64
Grams total	26.318	44.225	69.245	45.62
Per cent of administered drug	97.47	88.45	99.17	99.17
Per cent conjugated	0.78	4.4	1.3	2.1

his bowels and bladder. The total daily stools and urines were collected thereafter and analyzed for free and total sulfonamide content until the drug was no longer detectable in the excreta.

The technique of these determinations was as follows: The total daily feces were emulsified in 1,000 to 2,000 c.c. of tap water and filtered several times through cotton gauze. An aliquot portion was diluted anywhere from 1:3 to 1:500 with tap water depending on the concentration of sulfonamide anticipated. This solution was tested for its sulfonamide content by the method

of Bratton and Marshall.[5] Appropriate dilutions of samples of the total daily urine were similarly analyzed for their sulfonamide content.

The results of the excretion studies on each subject are shown in Table II. Although most of the drug was excreted during the course of administration, detectable concentrations were found in the excreta for two or three days after sulfacarboxythiazole had been discontinued. The percentages of the drug recovered in the stools were between 83.4 and 92.1 per cent, of which 3.4 per cent or less was in the conjugated form. In the urines the amounts recovered varied from 2.7 to 7.1 per cent, with 16.5 to 22.3 per cent occurring as the conjugated form. The total amounts recovered from both excreta ranged from 88.5 to 99.2 per cent of the amounts administered. The blood concentrations are reported in Table I.

BACTERIOLOGIC STUDIES

As part of the investigation of sulfacarboxythiazole, a study of its antibacterial activity on the intestinal flora of twenty subjects was made. The number of colonies of coliform organisms was used as an index of the intestinal population. Either the total amount of feces passed in twenty-four hours or a single specimen was weighed and then suspended in 1,000 to 2,000 c.c. of sterile isotonic salt solution. A series of tenfold dilutions were then made of this suspension. These diluted specimens were mixed in pour plates with tryptose-phosphate agar containing 5 mg. per 100 c.c. of para-aminobenzoic acid. The plates were incubated overnight at 37° F., after which time the colonies were counted and the number calculated for each gram of feces. The number of coliform colonies per gram present on the day treatment with sulfacarboxythiazole was started and thereafter, including the first few days after treatment had been discontinued, are shown in Table III. In each instance a control culture taken the day before treatment revealed innumerable colonies of coliform organisms. Within twenty-four hours there was considerable decrease in the number of colonies. Thereafter the cultures were sterile or only occasional colonies were observed, until the drug was discontinued, after which the coliform population steadily increased.

CLINICAL STUDY

Fifty patients with diarrhea were treated with sulfacarboxythiazole. Shigellae were isolated in thirty patients and salmonellae in two, and in the case of eighteen patients the causative organism was undetermined. Forty patients were below the age of 12 years; the other ten were between 22 and 54 years of age. There was no evidence of any other disease in any of the patients in this series. The total daily dose employed for the children was calculated on the basis of 0.1 Gm. per kilogram of body weight. The initial dose was equivalent to one-half the total daily dose, and thereafter the calculated dose was divided into six doses administered every four hours. The only other therapeutic measures employed were restriction of diet and administration of fluids parenterally when necessary.

TABLE III. EFFECT OF SULFACARBOXYTHIAZOLE ON INTESTINAL BACTERIAL POPULATION USING COLIFORM ORGANISMS AS AN INDEX (NUMBER OF COLONIES OF COLIFORM ORGANISMS PER GRAM OF FECES)

	DAYS AFTER SULFACARBOXYTHIAZOLE STARTED																	
PATIENT	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
W. M.	24	22	0	2,400	I														
G. S.	1	0	0	5,000	120,000	I													
G. R.	2	1	0	1,200	2,000	1,800,000	I												
E. R.	4	0	0	1,120	440,000	6,800,000	I												
H. D.	344	28	0	600	780,000	2,700,000	I												
R. W.	1	0	2	12,000	680,000	4,000,000	I												
J. B.	13	4	0	560	160,000	900,000	4,000,000	I											
La. P.	5	2	2	4	60	I													
G. L.	100	6	0	0	670	140,000	900,000	I											
C. N.	280	59	50	0	2,400	800,000	2,000,000	I											
G. B.	40	13	9	0	2,900	658,000	4,840,000	I											
J. H.	26	3	0	31	3,260	634,000	1,520,000	I											
A. R.	1	0	0	0	0	15,800	995,000	I											
W. K.	54	0	0	0	0	2	960	1,600,000	5,200,000	I									
L. W.	6	0	0	0	0	0	420	1,200,000	I										
W. D.	3	0	0	24	0	7	108	17,000	1,900,000	I									
G. D.	60	44	21	2	0	0	960	1,600,000	4,740,000	I									
Lu. P.	2	0	0	0	0	0	0	9,500	380,000	650,000	1,980,000	I							
F. H.	167	63	0	0	0	0	0	1,000	66,000	3,100,000	I								
B. S.	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2,000	160,000	I

I, Innumerable colonies.
Heavy line indicates time at which sulfacarboxythiazole was discontinued.

TABLE IV. RESULTS OF SULFACARBOXYTHIAZOLE THERAPY IN FIFTY PATIENTS WITH DIARRHEA

CAUSATIVE ORGANISM	NUMBER OF PATIENTS	DAY OF TREATMENT ON WHICH RECTAL CULTURES BECAME FREE OF PATHOGENS				DAY OF TREATMENT ON WHICH DIARRHEA ABATED							DAY OF TREATMENT ON WHICH PATIENTS BECAME AFEBRILE			
		1	2	3	4	NO DIARRHEA	1	2	3	4	5	NO RESPONSE	AFEBRILE	1	2	3
Shigella dysenteriae																
Flexner	17	13	1		3		9	3	2	1		2	3	11	2	1
Newcastle	8	4	1	3		1	6	1					3	5		
Sonne	5	3	1	1		1	2					2	1	4		
Salmonellae	2	1			1	2							1	1		
Unidentified	18						11	3	1		1	2	4	12	1	1

The results of the administration of sulfacarboxythiazole in these fifty patients are shown in Table IV. In the group with specific infections, the causative organism could no longer be cultured from the stools after the fourth day of treatment and throughout the observation period of one to two weeks after treatment had been discontinued. Of the forty-six patients who had diarrhea, forty were free of this symptom by the fifth treatment day. For the other six patients the treatment was considered a failure because the diarrhea persisted in spite of the fact that sulfacarboxythiazole was administered for eight days. It is of interest, however, that the stools of four of the patients in the latter group contained pathogenic organisms before therapy was started and these organisms had disappeared from the stools by the fourth day of treatment. All of the thirty-eight patients whose temperatures were 99° F. or higher at the time treatment was begun were afebrile by the fourth day.

TOXICITY

Among the sixty persons who received the drug, six developed toxic reactions. Four patients developed diarrhea. Three of these were receiving amounts totaling 0.5 Gm. per kilogram of body weight per day and the other patient 0.25 Gm. per kilogram of body weight per day. The diarrhea stopped when the doses were decreased. One patient developed nausea and vomiting following the ingestion of the initial dose consisting of 14 tablets (7 Gm.) or 0.25 Gm. per kilogram of body weight. The sixth patient developed a nodular rash similar to those which occur following the administration of sulfathiazole. The rash disappeared promptly after the drug was discontinued.

No abnormal findings were observed in the blood or urine of any of the subjects, although these were examined at frequent intervals during the course of study.

DISCUSSION

Sulfacarboxythiazole is a poorly absorbed rather than a completely non-absorbable sulfonamide compound. In a series of 105 determinations, only occasionally was the total of the free and acetylated drug above 1 mg. per 100 cubic centimeters. Sulfacarboxythiazole was recovered from the urine of several subjects in amounts up to 7.1 per cent of the total amounts administered.

Additional evidence that sulfacarboxythiazole was occasionally absorbed in significant amounts was the nodular rash which was observed in the case of one patient. From these studies it appears, however, that almost all the drug is available in the active form in the bowel, since less than 3.4 per cent of the amounts recovered in the feces were in the conjugated form.

The results of this study of the absorption and excretion of sulfacarboxythiazole are consistant with those reported by Harris and Finland.[3] These investigators found that blood concentrations were usually below 1 mg. per 100 c.c. but that concentrations up to 2 mg. per 100 c.c. were occasionally observed. Whereas amounts of 83.4 to 92.1 per cent of the drug were recovered in the feces of our patients, Harris and Finland[3] found only 54.6 to 82.9 per cent. Furthermore, the amounts which they demonstrated in the urines of their group of patients were higher, 9 to 23 per cent, in comparison to recovery amounts of up to 7.1 per cent in our series. In both series only small amounts of the conjugated form of the drug were present in the feces.

TABLE V. RESULTS OF SULFADIAZINE THERAPY IN FIFTY PATIENTS WITH DIARRHEA

CAUSATIVE ORGANISM	NUMBER OF PATIENTS	DAY OF TREATMENT ON WHICH RECTAL CULTURES BECAME FREE OF PATHOGENS				DAY OF TREATMENT ON WHICH DIARRHEA ABATED					DAY OF TREATMENT ON WHICH PATIENTS BECAME AFEBRILE			
		1	2	3	4	1	2	3	4	NO RESPONSE	AFEBRILE	1	2	3
Shigella dysenteriae														
Flexner	24	15	6	2	1	10	8	1	3	2	4	12	6	2
Newcastle	8	6	1	1		7		1			6	1	1	
Sonne	3	2	1			1	1	1			1	2		
Unidentified	15					4	4	3	2	2	5	6	2	2

Sulfacarboxythiazole has been found to be satisfactory in the treatment of fifty patients with diarrhea. The results of treatment in this group were compared with the results (Table V) observed in fifty patients with similar illnesses who were treated with sulfadiazine prior to the period that sulfacarboxythiazole was employed. Stool cultures became negative within four days in both groups. Ninety-two per cent of the sulfadiazine-treated patients were free of diarrhea by the fourth treatment day, while 87 per cent were similarly improved when treated with sulfacarboxythiazole. This difference is not considered significant. Four sulfadiazine-treated and six sulfacarboxythiazole-treated patients continued to have diarrhea with negative stool cultures even though the drug was continued for eight days. Of the failures in the latter group, four improved on a course of sulfadiazine, although two of these patients had not responded at first to this drug. Sulfadiazine was ineffective in the two remaining patients who had failed to improve when they were given sulfacarboxythiazole. All patients in both groups who had elevated temperatures at the time treatment was started were afebrile within three days.

The only other clinical evaluation of this sulfonamide was reported by Harris and Finland.[3] They noted prompt recovery in five patients with dysentery following the administration of sulfacarboxythiazole.

In anticipation of the use of sulfacarboxythiazole in the preparation of patients for bowel surgery, stool cultures on plain agar plates were taken daily on twenty subjects. The prompt disappearance and continued absence of most of the intestinal flora from the stools while the drug was continued would make it appear that the drug is effective as an intestinal antiseptic.

SUMMARY AND CONCLUSIONS

1. Sulfacarboxythiazole (2-sulfanilamido-5-carboxythiazole) is a poorly absorbed sulfonamide when given orally. Blood concentrations were usually below 1 mg. per 100 cubic centimeters. The excretion of the drug in the stools varied between 83.4 and 92.1 per cent of the amount ingested, while the urinary excretion ranged from 2.7 to 7.1 per cent of the oral dose.

2. Sulfacarboxythiazole gave satisfactory results in forty-four out of fifty patients with diarrhea. The results of this treatment compare favorably with the results of sulfadiazine therapy in a similar group of fifty patients.

3. Six patients developed toxic reactions from sulfacarboxythiazole. Gastrointestinal symptoms developed in five patients, in four of whom the large doses administered were undoubtedly responsible. A sixth patient developed a nodular skin rash.

4. Sulfacarboxythiazole appears to be a useful sulfonamide in the treatment of enteric infections caused by sulfonamide-sensitive bacteria and deserves a trial in the preparation of patients who are to undergo bowel surgery.

We wish to thank Miss C. Barbara O'Neil for technical assistance.

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THE CONCENTRATION OF STREPTOMYCIN IN DOG BILE

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STREPTOMYCIN has been found to be effective against the intestinal types of microorganisms, including *Escherichia coli*, and the dysentery and typhoid organisms.

Reimann, Elias, and Price[1] and the Committee on Chemotherapeutic and Other Agents of the National Research Council[2] have used streptomycin in the treatment of patients with typhoid fever. No evidence was obtained proving that the duration or mortality rate was influenced because too few cases were studied.

Rutstein and associates[3] studied the effect of streptomycin on three typhoid carriers. They found that 150,000 units given intramuscularly every three hours produced effective blood levels but did not affect the typhoid bacilli in the stool. Following the oral administration of 31,000 units every three hours, a definite transitory bacteriostatic effect on the typhoid bacilli in the stool was obtained. Typhoid bacilli grow well in bile, and organisms remaining in the gall bladder and in the bile may be responsible for the carrier state. However, Rutstein and his associates did not simultaneously use the oral and parenteral routes of administration.

The results of studies on the excretion of streptomycin in the bile of the rabbit and the human patient have been reported. Stebbins, Graessle, and Robinson[4] found that rabbits excreted from 5 to 10 per cent of intravenously administered streptomycin. Zintel and co-workers[5] gave 600,000 units intravenously to two human patients and obtained streptomycin levels in the bile of 8 units per cubic centimeter. Heilman and associates[6] gave one patient 100,000 units intramuscularly every three hours for five days. They obtained bile concentrations as high as 13 units per cubic centimeter. The serum level at the same time was 6 units per cubic centimeter. It was concluded that the liver could concentrate the drug. Zaslow,[7] in a preliminary report, administered streptomycin to patients before cholecystectomy and obtained considerable amounts of the antibiotic in gall bladder bile, if the cystic duct was patent. In the case of an obstructed cystic duct, no streptomycin was found in the gall bladder. Even though the drug remained in the gall bladder for a considerable length of time, he found no evidence of absorption or concentration. In intrahepatic and in obstructive jaundice, no antibiotic agent was excreted in the bile. In patients with normal livers, considerable amounts of streptomycin appeared in the hepatic bile; however, the level of streptomycin activity in the bile was less than that in the blood, indicating that the liver did not concentrate this anti-

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biotic agent. Zaslow did not, however, administer sufficient streptomycin to produce a high level in the serum and bile.

In this investigation we attempted to answer the following questions: (1) Can high levels of streptomycin be obtained in hepatic bile? (2) Can high levels of streptomycin be obtained in gall bladder bile? (3) Is streptomycin concentrated by the normal gall bladder?

EXPERIMENTAL

Five dogs were anesthetized with pentobarbital. The common bile duct was cannulated, and the cystic duct was clamped off.

During the course of twenty minutes, streptomycin was given by intravenous drip in amounts ranging from 187,000 to 375,000 units. In Table I are given the results for a typical dog.

In an attempt to ascertain whether the liver would concentrate the drug, one dog was treated similarly, and simultaneous blood and hepatic bile levels were obtained. The results are shown in Fig. 1.

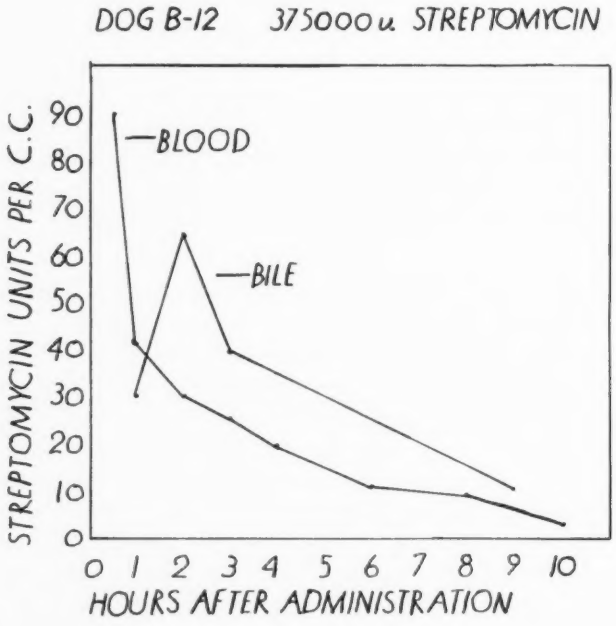

Fig. 1.

The concentration of streptomycin in gall bladder bile was investigated in six unanesthetized dogs which received the drug by intravenous drip and which were anesthetized one hour after the administration of the drug. Three of the dogs were in a fasting state, and three others received a meal of egg yolks and cream five hours before the experiment. The results are shown in Table II.

Twelve more dogs received streptomycin intramuscularly while in the fasting state. Three were given a single injection at 7 P.M. on the first day. At 11 A.M. on the second day, the animals were anesthetized and their gall bladder

content was removed. The other nine dogs received a total of seven injections each, four at three-hour intervals beginning at 10 A.M. on the first day and three more injections beginning at 10 A.M. on the second day. The animals were anesthetized at 11 A.M. on the third day. The results are shown in Table III.

TABLE I. DOG A-2 (187,500 UNITS OF STREPTOMYCIN)

MINUTES AFTER STARTING INTRAVENOUS DRIP	BILE VOLUME (C.C.)	STREPTOMYCIN CONCENTRATION IN BILE (UNITS PER C.C.)	TOTAL STREPTOMYCIN (UNITS)
Control	3.7	0	0
30	4.3	6	26
60	3.0	50	150
120	4.6	135	621
165	2.9	108	313

TABLE II.

DOG	STREPTOMYCIN (UNITS)	MEAL	STREPTOMYCIN CONCENTRATION IN BILE (UNITS PER C.C.)
B-1	50,000	None	1.2
B-2	50,000	None	0.6
B-3	50,000	Egg yolks and cream	0
B-6	50,000	Egg yolks and cream	0
B-10	375,000	None	22
B-11	375,000	Egg yolks and cream	25

TABLE III.

NUMBER OF DOGS	NUMBER OF INTRAMUSCULAR INJECTIONS	TOTAL AMOUNT OF STREPTOMYCIN RECEIVED (UNITS)	AVERAGE VOLUME OF GALL BLADDER BILE (C.C.)	AVERAGE STREPTOMYCIN CONCENTRATION (UNITS PER C.C.)
1	1	100,000	11	<2
2	1	160,000	20	2
3	7	140,000	11	<2
3	7	280,000	17	2.5
3	7	420,000	10	4.5

DISCUSSION

The results on the five dogs with a cannula in the common duct indicate that a maximum concentration of streptomycin in hepatic bile was reached in two or three hours. The highest level obtained, 135 units per cubic centimeter, was in Dog A-2 which received 187,500 units by intravenous drip in twenty minutes. Small amounts of streptomycin were still present in the bile after ten hours. The total amount excreted in the bile was only a fraction of a per cent of the amount administered.

The graph in Fig. 1 shows that two hours after starting the intravenous drip the serum concentration was 30 units of streptomycin per cubic centimeter while the concentration in hepatic bile was 64 units per cubic centimeter, indicating that the normal liver of the dog concentrates streptomycin.

The results in Table II indicate that 50,000 units given intravenously are inadequate to produce high levels in the gall bladder bile, irrespective of whether

a meal of egg yolks and cream was given or not. With 375,000 units, adequate levels of streptomycin in the gall bladder were obtained. Here also a meal of egg yolks and cream made little difference.

The results on the dogs which received streptomycin intramuscularly indicate that: (1) A single intramuscular injection of the drug will produce about as high a concentration in the gall bladder as will the same quantity divided into several doses; and that (2) the gall bladder does not appear to concentrate streptomycin since repeated intramuscular injections do not produce nearly as high a level in the gall bladder bile as does smaller doses given intravenously during the course of twenty minutes.

SUMMARY

1. Studies on streptomycin in hepatic and gall bladder bile of twenty-three dogs were conducted.

2. High levels of streptomycin were observed in both hepatic and gall bladder bile when relatively large doses were given slowly intravenously.

3. The liver was observed to concentrate streptomycin in one experiment.

4. The gall bladder does not concentrate streptomycin. The salt of streptomycin, being soluble in saline solution, is apparently absorbed along with water and electrolytes when the bile is concentrated by the gall bladder.

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CUTANEOUS TEST WITH COCCIDIOIDIN

Review of the Literature and Report of a Series in Texas

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SINCE the original description of coccidioidal granuloma in South America by Wernicke[1] and Posada[2] in 1892 and in North America by Rixford and Gilchrist[3, 4] in 1894 and 1896, great advances have been made in our understanding of the nature of the disease,[5] of its pathologic,[6, 7] clinical,[8-10] and epidemiologic[11] features, and especially of the diagnostic criteria involved. Among these criteria, one of the most objective and, therefore, reliable is the specific skin test with coccidioidin, which is regularly used in ruling coccidioidomycosis in or out in cases having a clinical picture compatible with the disease. Although the test is widely used, there has been to date no comprehensive review of the literature pertaining to it; it is the purpose of the present report to fill this need and to present the results of a series of coccidioidin tests performed in Dallas, Texas.

Cooke,[12, 13] in 1914 and 1915, first undertook investigations sighted at the detection of agglutinins, precipitins, and complement-fixing substances in the serum of patients with coccidioidal granuloma, and her work gave the impetus to other workers to investigate cutaneous reactions in this disease. Using antigens prepared from the mycelial growth on solid culture mediums and from the sporelike bodies present in the exudate from lesions, Cooke concluded that these antigens could not be used to demonstrate specific complement-fixing substances or agglutinins in the blood serum of patients nor did they produce any specific skin reactions. She found precipitins in serum diluted 1:160 and mixed with an extract of dried cultures. These studies were continued in 1916 by Cummins and Sanders[14] who reported that, although they could not demonstrate agglutinins, precipitins, or complement-fixing substances in the serum of patients and animals with the disease, the concentrated filtrate from heated and unheated broth cultures caused a cutaneous reaction in some of their infected rabbits and to a lesser degree in normal rabbits. Davis[15] obtained complement-fixing reactions with a concentrated culture as antigen and reported an intradermal skin reaction with killed homologous organisms definitely more marked than that obtained following the injection of a similar antigen made from cultures of Sporothrix, Blastomyces, or agar. Hirsch and Benson[16] concluded from the known histologic reactions to *Coccidioides immitis*, with its similarity to the specific reaction to *Mycobacterium tuberculosis* and its lack of identity with a nonspecific foreign body reaction, that some soluble substance is produced by *C. immitis* which resembles,

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in some respects at least, the tuberculin produced by the tubercle bacillus and that skin reactions can be obtained in patients and animals infected with *C. immitis* which resemble those with tuberculin in tuberculous hosts. Using a modification of the medium used by Braun and Cahn-Bronner[17] in their studies of general bacterial physiology, they[16] found that growths of *C. immitis* in effect liberate a soluble specific substance causing skin reactions in infected animals and patients. Hirsch and Benson were the first workers to study the properties of this soluble specific substance. They found that it is not destroyed by heating to 80° C. for thirty minutes and that it is flocculated by electrodialysis. In 1928, da Fonseca and de Area Leão[18] gave the first accurate description of a positive coccidioidin skin test. They pointed out that sensitive specific reactions are obtained to filtrates of *C. immitis* injected intradermally and that these reactions consist of an erythema accentuated at the inoculation site and a papular erythematous induration appearing several hours after injection and gradually increasing to a maximum in twelve to twenty-four hours. They further observed that the test may be accompanied by temperature elevation and focal reaction in patients with coccidioidal granuloma.

Beck, Traum, and Harrington[19] further studied the problem in animals and raised the question of possible cross reactions with tuberculosis, reporting weakly positive tuberculin reactions in animals infected with *C. immitis*. Hurwitz, Young, and Eddie[20] also reported positive coccidioidin reactions in patients having no known coccidioidal infection. However, Gifford, Buss, and Douds,[21] in an extensive survey in Kern County, California, and in studies of patients with San Joaquin fever, showed specificity of the coccidioidal reaction and were pioneers in reporting a high incidence of positive tests in individuals living in the San Joaquin valley. Dickson and Gifford,[22] in 1938, following the preliminary work of Jacobson,[23] showed that cutaneous sensitivity to coccidioidin occurs six to seven days after the inoculation of guinea pigs and rabbits by chlamydospore inhalation. These authors[22] stated that the active antigenic substance was not identified, questioning by implication the work of Hirsch and Benson[16] (vide supra). They also made the observation that patients with valley fever react with a violent necrotizing response to the intradermal injection of 0.1 c.c. of filtrate, as advocated by Jacobson in a study the results of which were further corroborated by Dickson[24, 25] in the same year, when he showed that the reaction of valley fever patients to coccidioidin was much more violent than that of patients with coccidioidal granuloma. This finding repudiated the preliminary impression of da Fonseca and de Area Leão[18] (vide supra).

Kessel,[27] in 1939, showed that in the final stages of coccidioidal granuloma four weeks before death the skin test is negative but that other active cases of coccidioidal granuloma prior to the final stages give a positive skin reaction. He also demonstrated that patients with tuberculosis as a rule give negative reactions, that guinea pigs experimentally inoculated with *Myco. tuberculosis* give a negative coccidioidin skin test and a positive

tuberculin test, and that guinea pigs experimentally inoculated with *C. immitis* give a positive coccidioidin skin test and react negatively to tuberculin tests. By these experiments, Kessel furnished objective evidence to support the presumedly high degree of specificity inherent in the coccidioidin skin test, a specificity which had already been suspected by Dickson.[28]

The investigation of coccidioidin sensitivity in children and the correlation of positive tests with the existence of a syndrome consisting of fever, cough, aching, anorexia, sore throat, and pleural pain followed in from a few days to two and one-half weeks by erythema nodosum and positive sputum culture at the time of coccidioidin positivity, was undertaken in 1939 by Faber, Smith, and Dickson,[29] and their results checked closely with those anticipated on the basis of previous studies. In the following year, Smith[30] further studied the epidemiologic aspects of coccidioidomycosis by performing intradermal tests on healthy volunteers who had had clinical San Joaquin valley fever. These tests were all strongly positive; however, sensitivity to the antigen acquired from clinical infection could not be differentiated from sensitivity due to reinfection, since the subjects were residents of an endemic area. An isolated example suggesting strongly that sensitivity acquired during initial contact with the organism is permanent was cited. Smith's results indicated also that sensitivity to coccidioidin is established in from two to seventeen days following the onset of symptoms.

The geographic distribution of endemic centers of coccidioidal infection as determined by cutaneous test is listed by Davis, Smith, and Smith[11] and according to these writers includes, besides the San Joaquin valley, Southern California, Arizona, Texas, and probably New Mexico and Mexico. Sporadic cases are also said to have been reported from Europe and from the Chaco region of South America. A new endemic area at Camp Roberts, California, was described in 1942 by Shelton,[31] who, using the antigen supplied by Dr. C. E. Smith (the same antigen as was used in the series reported in the present paper), showed that of 736 soldiers who came from other parts of the United States to Camp Roberts fourteen became infected during three months' residence there. His original series of tests on 888 soldiers constituting a heterogeneous group chiefly from the Middle West (Illinois, Missouri, Nebraska, Arkansas, and Texas) resulted in eleven positive reactions or 1.2 per cent incidence of positivity. In the same year (1942), Aronson, Saylor, and Parr,[32] working in the endemic area of Arizona, demonstrated presumptively that the occurrence of calcified pulmonary nodules may be due to pulmonary infection with *C. immitis,* using the coccidioidin skin test and the tuberculin test as criteria for evaluation of the roentgenograms. Previously, in 1939, Phillips[33] had drawn attention to possible endemicity of coccidioidal infection in Phoenix, Arizona, but he quoted a series too small to constitute more than suggestive evidence of such endemicity.

Smith[34] has interpreted the skin reaction to coccidioidin as a nonspecific allergic manifestation of a specific sensitization. He pointed out that the sensitivity to coccidioidin becomes established in clinical cases of primary

coccidioidomycosis just before the appearance of the characteristic erythema nodosum, which must be present in order to establish a diagnosis of valley fever. He points out, further, that this eruption may represent either an undulating allergy or an effect of a circulating antigen in a hypersensitive person.

Peers, Holman, and Smith[35] further demonstrated the usefulness of the cutaneous reaction to coccidioidin in differentiating coccidioidomycosis from pulmonary tuberculosis. In an epidemic of primary pulmonary coccidioidomycosis comprising seventy-five cases reported by Goldstein and Louie,[36] there was 100 per cent correlation between the clinical entity and the skin test. In a recent paper, Denenholz and Cheney[37] stress the diagnostic significance of the coccidioidin skin test, pointing out that a positive test probably has the same meaning in coccidioidomycosis as a positive reaction to tuberculin has in tuberculosis; it indicates that the patient has, or has had in the past, a coccidioidal infection, either clinical or subclinical, and does not necessarily imply that a positive reactor's present illness is due to infection with *C. immitis*. A negative coccidioidin skin test generally excludes the possibility of a coccidioidal infection, with only two possible exceptions, namely, the theoretic possibility that a patient with an active coccidioidal infection due to a strain not present in the antigen might react negatively to the test and the actual, but rare, possibility of negative reaction in a patient with severe disseminated coccidioidomycosis in a late anergic phase.

Emmons and Ashburn[38] have demonstrated that there is some cross sensitivity between *C. immitis* and a fungus isolated by them from rodents in Arizona which they have named *Haplosporangium parvum*. It may be that this cross sensitivity is responsible for the borderline positive reactions to coccidioidin commonly encountered in persons living in some areas in the Middle West, as pointed out by Smith.[34] In any case, the test may legitimately be considered highly specific in the overwhelming majority of cases, indicating coccidioidal infection, past or present.

REPORT OF A SERIES IN DALLAS, TEXAS

There is no report in the literature of any series of coccidioidin skin tests performed in Texas; yet from other series the impression has been gained that Texas possibly represents an endemic focus of coccidioidal infection. The present paper presents the results of an investigation undertaken in Dallas, Texas, with the purpose of determining the incidence of positive coccidioidin tests in an unselected series as far as probable or possible coccidioidin reactivity was concerned. In this study, 413 persons were tested. The subjects comprised 98 medical students, 102 student nurses, 202 patients on the wards of Parkland Hospital, and a miscellaneous group of 11 additional individuals. The following personal data were obtained from each subject before the test was performed: age, previous occupations, history of erythema nodosum, previous coccidioidin test with result, results, if known, of previous x-ray examination of the chest, home state, other places of residence, and places of travel.

The coccidioidin which was used in the performance of all the tests in the series was obtained from Dr. C. E. Smith of the Department of Public Health at Leland Stanford Junior University. The antigen, as supplied, was prepared by growing ten strains of fungus coccidioides in the Bureau of Animal Industry medium, the formula used for making tuberculin modified only in the use of 2.5 per cent glycerine rather than stronger concentrations. After the fungus had been grown for two months, it was Berkefeld filtered and merthiolate was added to a final concentration of 1:10,000. This material was subjected to sterility tests, animal inoculation, and tests on human beings of known sensitivity. The dilution used was 1:100 in sterile physiologic saline, and the tuberculin syringes used had never been used for any other procedure. Each needle used was employed for several consecutive tests without resterilization.

Each test consisted of the intradermal injection of approximately 0.1 c.c. of the diluted antigen into the skin on the volar aspect of the right forearm. Special care was taken that a wheal was raised at the site of injection in every member of the series, that is, that a true intradermal test was done. Each test was read in twenty-four and forty-eight hours. Any test showing a palpable induration at the injection site was considered to be a positive test; all others were recorded as negative. Grading of the tests was not attempted on the basis of standards any more minutely defined than those described.

On nine and eight, respectively, of the positive reactors to coccidioidin, simultaneous tuberculin and histoplasmin tests were done by McGolrick and Moore[39] as a part of an investigation undertaken to correlate skin tests with tuberculin and histoplasmin with thoracic roentgenogram evidence of pulmonary calcific nodules; the results are mentioned in the next section.

RESULTS OF THE TESTS

Of the 413 subjects, eleven (or 2.66 per cent) were positive reactors by the criteria which have been defined. This incidence of positivity is somewhat higher than that found by Shelton, whose group was, as noted, a homogeneous one taken chiefly from the Middle West, indicating that the incidence of sensitivity to coccidioidin is slightly greater than that encountered in Camp Roberts, California, prior to the establishment of endemicity there. No significant correlation between vocation and skin test positivity was obtained in the positive reactors; in fact, all but two of the reactors were persons whose occupations kept them indoors and hence were not especially liable to inhalation of chlamydospores of *C. immitis.*

Tuberculin tests were done on nine of the positive reactors as part of a series run simultaneously with the present one by McGolrick and Moore[39] on the nurse and medical student groups. To a 1:1000 dilution of tuberculin (O.T.), one subject reacted positively and one gave a doubtful reaction, all the remainder being negative; whereas in the case of a dilution of 1:100, there were one positive and three doubtful reactors. In histoplasmin tests done by the same workers on eight of the positive coccidiodin reactors, three were found to be positive. Only one of these simultaneously tested subjects showed a positive reaction to

both dilutions of tuberculin, to histoplasmin, and to coccidioidin, although another subject who gave a clearly positive reaction to coccidioidin and histoplasmin also reacted to tuberculin in both dilutions, though doubtfully according to the criteria adopted by McGolrick and Moore. In summary, the majority of of the coccidioidin-positive subjects in this series were found to be nonsensitive to antigens of tuberculosis and histoplasmosis, two common causes of pulmonary calcification.

Of the positive reactors, only two had been previously tested with the antigen, and their reactions had been positive, corroborating the work previously quoted that sensitivity to coccidioidin, once acquired, tends to persist.

The states of residence of the coccidioidin positive reactors were investigated, and in cases in which the person in question had been a resident at various times of several states, each of those states was recorded. It was found that Texas was at one time, past or present, the state of residence of 81.8 per cent of the individuals studied; California was the next most frequently encountered state of residence (45.4 per cent), and Arizona was the third in frequency with 36.3 per cent. Some significance can thus be attached to the fact that most of the positive coccidioidin reactors had at one time lived in one of several well-known endemic areas. The fact that the overwhelming majority had lived in Texas is, of course, a factor of the locale in which the survey was undertaken. Territories in which the members of the positive reactor group had traveled were also surveyed, and only periods of travel were included in which the individuals had remained in the various locales for at least two weeks. That three had traveled in Mexico is of interest in the light of the suggestive evidence that there exists lurking endemicity in that country,[10] but a definitive survey of Mexico has not appeared in the indexed literature to date.

Most of the positive reactors were white, although approximately as many Negroes as Caucasians were tested. This finding may be partially explained by the fact that the Negro population in the area surveyed have a tendency not to travel outside of their site of origin. It is of significance to note that all but three of the positive reactors had traveled away from the place of study, some quite widely.

The ages of the positive reactors ranged between 18 and 40, and most of the subjects fell in the age group of 18 to 20. This cannot, however, be said to demonstrate any significant relationship between age and incidence of coccidioidin positivity for this series.

SUMMARY

1. The literature pertinent to the cutaneous test with coccidioidin is reviewed.

2. A report is made of a group of 413 subjects in Dallas, Texas, comprising nurses, medical students, and general hospital patients who were subjected to intracutaneous coccidioidin tests. In this group, an incidence of 2.66 per cent positive reactors was encountered.

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METHOD OF ASSAYING STEROIDS AND ADRENAL EXTRACTS FOR PROTECTIVE ACTION AGAINST TOXIC MATERIAL (TYPHOID VACCINE)

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THE protective action of adrenal extract against the acute toxic effects of typhoid vaccine and the chronic toxic effects of *Staphylococcus aureus* vaccine in adrenal deficient rats was reported by Hartman and Scott[1] in 1932. The results of Ettelson[2] confirmed their protective power against typhoid vaccine intoxication and demonstrated the relative ineffectiveness of desoxycorticosterone acetate. The possibility of using these observations as an assay method for determining what factor or factors in adrenal extract are responsible for protection against toxic materials was evident. The following method has been evolved and appears to give constant and reproducible results.

METHOD

Male rats (Sprague-Dawley strain) weighing between 80 and 120 grams each were adrenalectomized under ether anesthesia, and after an interval of at least five days the animals were ready for the test. The diet consisted of Purina dog chow. Nine-tenth per cent sodium chloride was offered for drinking. Room temperature was maintained between 80 and 85° Fahrenheit.

The minimum lethal dose (M. L. D.) of typhoid vaccine (Lederle combined TAB vaccine), which would cause 100 per cent mortality within twenty-four hours when administered intraperitoneally, was determined on some of the adrenalectomized rats maintained on 0.9 per cent sodium chloride. The test dose adopted was 1.33 M. L. D. The adrenalectomized animals not used for estimation of the M. L. D. were used for testing the protective power of the material to be assayed. Extracts were administered subcutaneously at 9:00 A.M. and 5:00 P.M. if in aqueous or alcohol-aqueous solution, once a day at 9:00 A.M. if in oil. They were given for three days. Immediately after the last morning injection of extract, 1.33 M. L. D. of typhoid vaccine were administered intraperitoneally. The percentage of animals surviving twenty-four hours later was recorded. The surviving animals were then placed on plain drinking water. Those alive after twenty-one days were sacrificed and searched for remnants of adrenal tissue. If any adrenal tissue was found, the observation on the animal was not used in calculating the protective power of the extract.

One toxic protection unit is defined as the amount of material, in terms of any convenient unit, per twenty-four hours which will protect 90 per cent of adrenalectomized rats against 1.33 M. L. D. of typhoid vaccine, equivalent in protective power to 0.28 mg. 11-dehydrocorticosterone acetate.

RESULTS

The percentage of rats surviving twenty-four hours after 1.33 M. L. D. typhoid vaccine intraperitoneally, when treated with various amounts of adrenal extract, is shown in Fig. 1. When 1 c.c. per twenty-four hours of whole adrenal extract (Upjohn) was given, 90 per cent of the animals were protected; this showed that the extract, according to the foregoing definition, contained 1 toxic protection unit per cubic centimeter.

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The protective power of the extract was nearly completely destroyed when the extract was alkalinized to pH 11.0, boiled for ten minutes, and the pH readjusted to pH 7.0. The equivalent of 2 c.c. of extract after this treatment showed no protective effect. One cubic centimeter of untreated extract protected 90 per cent of the animals.

The potencies of four extracts were determined by the toxic protection test and compared with assay values estimated by the Ingle muscle-work test. They showed 1.0, 2.5, 2.2, and 10.0 toxic protection units per cubic centimeter, respectively, as compared with 1.0, 2.8, 5.0, and 11.5 units by the muscle-work test. One unit by the Ingle muscle-work test is equivalent to the effect produced by 0.2 mg. 11-dehydro-17 hydroxycorticosterone.

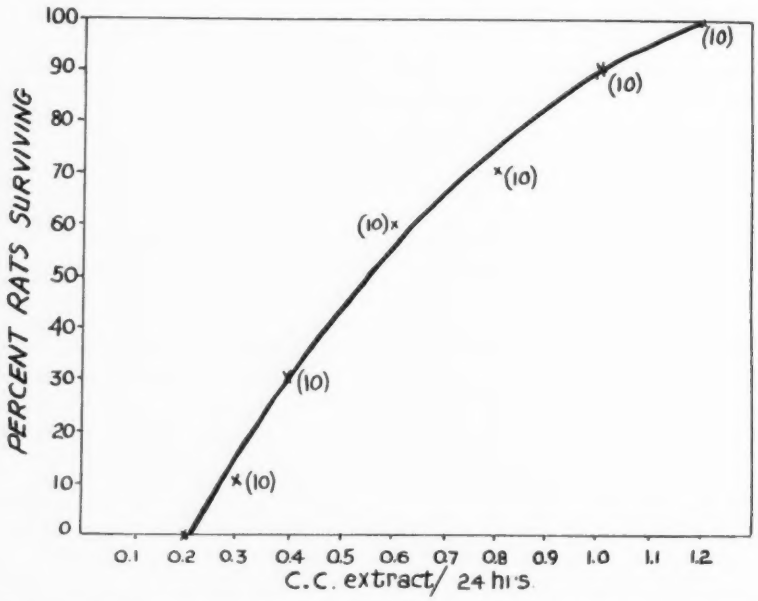

Fig. 1.—Protective power of whole adrenal extract (Upjohn) against 1.33 M. L. D. typhoid vaccine in adrenalectomized rats.

When the rats were injected with between 3 and 10 mg. of desoxycorticosterone acetate per day, 75 to 85 per cent survived. Summarized in Fig. 2 are the results obtained when desoxycorticosterone acetate was administered once a day in peanut oil. When it was dissolved in propylene glycol and injected twice daily, the protective action at the lower dose levels was essentially the same as that when dissolved in oil. When 5.0 mg. or more, involving the injection of 0.5 c.c. or more of propylene glycol, were given, less protection was observed than when the material was in oil solution.

Free desoxycorticosterone when administered in oil showed a protective power of 1 toxic protection unit per 2 milligrams. The protection of 90 per cent of the animals with 2 mg. of free desoxycorticosterone per day and 100 per cent of the animals with 3 mg. was interesting, since not more than 85 per cent of

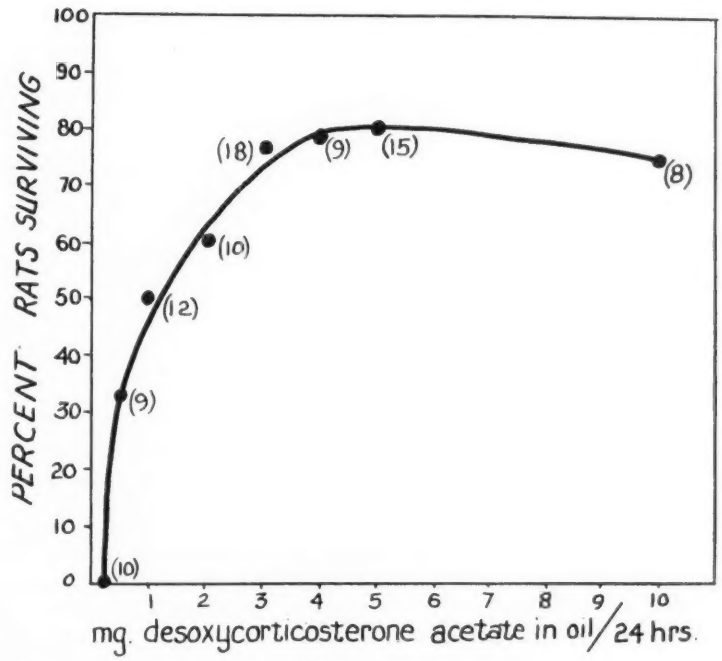

Fig. 2.—Protective power of desoxycorticosterone acetate in peanut oil against 1.33 M. L. D. typhoid vaccine in adrenalectomized rats.

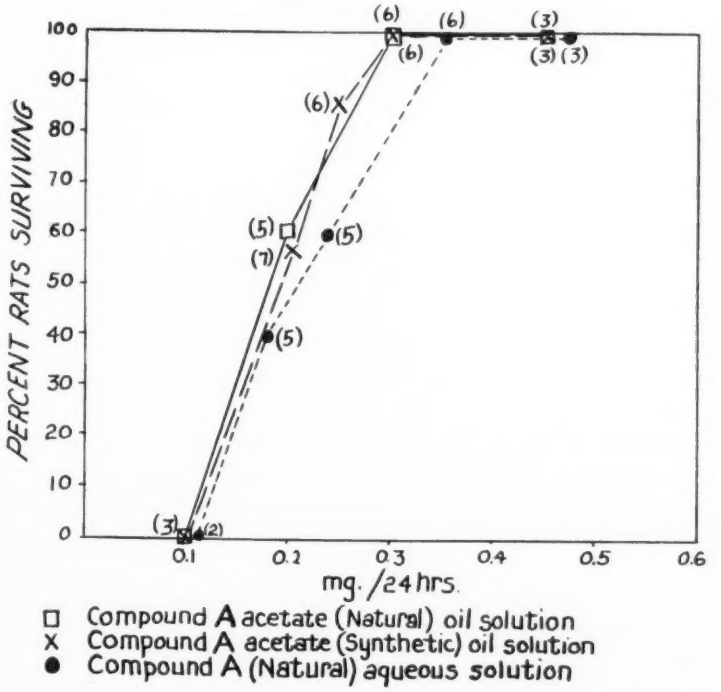

Fig. 3.—Protective power of compound A and compound A acetate against 1.33 M. L. D. typhoid vaccine in adrenalectomized rats.

the animals were protected when the acetate was employed in doses from 2.5 to 10 mg. per twenty-four hours. The slightly greater solubility of desoxycorticosterone over desoxycorticosterone acetate may account for the greater protective action.

Corticonebenzoate protected 100 per cent of the test animals when administered in amounts of 0.75 mg. per day, indicating slightly more than 1.3 toxic protection units per milligram.

A series of experiments was carried out using 11-dehydrocorticosterone and 11-dehydrocorticosterone acetate prepared from adrenal tissue and 11-dehydrocorticosterone acetate synthetically prepared. The protective power of natural and synthetic compound A acetate in oil was essentially the same (Fig. 3), being 3.6 units per milligram. Compound A in aqueous solution administered twice daily showed a protective power of 3.1 units per milligram. The more constant level of the compound in the blood obtained when the hormone was given in oil probably accounts for the apparently greater potency of the compound A acetate. When administered in doses of 3.0 mg. per day, 11-ketoprogesterone protected 100 per cent of the animals treated; while 0.3 mg. per day protected 54 per cent. Progesterone in the same amounts gave no protection.

TABLE I

COMPOUND	TOXIC PROTECTION (UNITS PER MG.)
11-dehydro-17 hydroxycorticosterone	5.5
11-dehydrocorticosterone acetate (natural)	3.6
11-dehydrocorticosterone acetate (synthetic)	3.6
11-dehydrocorticosterone (natural)	3.1
Corticonebenzoate	1.3
11-ketoprogesterone	More than 0.5, less than 3.0
Desoxycorticosterone	0.5
Desoxycorticosterone acetate	Less than 0.1
Acetoxypregnenolone	Less than 0.1
Methyl androstenediol	Less than 0.1
Progesterone	Less than 0.3

Acetoxypregnenolone and methyl androstenediol in doses as large as 10 mg. per day failed to show any protection.

Compound E, 11-dehydro-17 hydroxycorticosterone, showed 5.5 toxic protection units per milligram. This was a little more potent than any other compound tested.

In an attempt to determine whether the maintenance of a normal blood glucose level was the effective mechanism of protection against the typhoid toxin, some of the adrenalectomized animals suitable for testing were injected subcutaneously with 2 c.c. of 5 per cent glucose a half hour before the administration of 1.33 M. L. D. of typhoid toxin. They were injected with a similar amount of glucose solution every two hours or each hour thereafter. Half of the animals, when they began to become moribund, were bled for blood glucose determination. The remainder were kept for the lethal studies. All animals were dead within eight hours following the administration of toxin. The blood glucose levels determined on animals receiving injections each two hours were

70, 95, 65, 105, and 88 mg. per 100 c.c., respectively; the blood glucose levels of those receiving hourly injections were 65, 105, 80, 120, 70 mg. per 100 cubic centimeters; respectively.

DISCUSSION

The protective power of adrenal extract, 11-dehydrocorticosterone, 11-dehydrocorticosterone acetate (natural) 11-dehydrocorticosterone acetate synthetic, corticonebenzoate, 11-dehydro-17 hydroxycorticosterone, and desoxycorticosterone against the toxic effects of typhoid vaccine when administered to adrenalectomized rats closely paralleled their relative potencies as judged by their effects on carbohydrate metabolism. The fact that 11-ketoprogesterone showed toxic protective power was interesting. Data on its effect on carbohydrate metabolism will be published later. All of the compounds with an oxygen at C-11 showed high toxic protection power. The maintenance of normal or near normal blood glucose levels by injection at frequent intervals of 5 per cent glucose in saline was not, however, effective alone in prolonging the life of the typhoid vaccine injected animals. It appears that some other factors besides normal blood glucose must be active in protecting against the typhoid vaccine.

These results suggest the possible usefulness of adrenal extract and certain crystalline preparations of adrenal origin in combating acute toxic reactions. It is possible that the administration of large amounts of these substances would protect patients during the most acute phases of a toxic disease, as in typhoid or typhus fever, especially until the amount of hormone produced by the patient's own adrenal would be adequate for protection.

Further animal experiments are being carried out to determine whether the compounds that have been found effective against typhoid toxin are also able to protect against other types of bacterial toxin.

SUMMARY

A method has been described for determining the relative protective power of adrenal extracts and steroids against the toxic effects of typhoid vaccine on adrenalectomized rats. Of eleven crystalline compounds studied 11-dehydro-17 hydroxycorticosterone was the most potent. All of those compounds having an oxygen at C-11 exhibited marked power to protect against typhoid vaccine. The natural and synthetically prepared 11-dehydrocorticosterone acetate (compound A acetate) showed identical potency.

We wish to thank Dr. M. H. Kuizenga of the Upjohn Company, for generous supplies of various adrenal extracts; Dr. E. C. Kendall of the Mayo Clinic, for the natural and synthetic compound A acetate, the compound E, and compound A; Dr. Irwin Schwenk of the Schering Corporation, for the desoxycorticosterone acetate; and Dr. B. E. Lowenstein of our own laboratory, for the corticonebenzoate and the 11-ketoprogesterone used in these studies.

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TRIDIONE: A NEW ANTICONVULSANT DRUG

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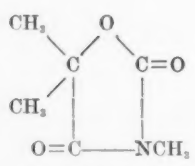
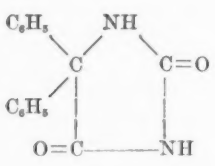
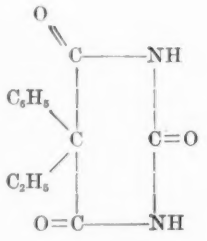
TRIDIONE (3,5,5-trimethyloxazolidine-2,4-dione) was first synthesized by Spielman[1] in a systematic search for new analgesic drugs. Pharmacologic studies indicated that the compound possessed analgesic properties,[2] and this was later substantiated by clinical investigation. During further studies in animals it was found that Tridione suppressed convulsions caused by other drugs in doses which produced little or no neurologic deficits. This led to a more extensive study of the anticonvulsant properties of the drug by Richards and Everett[2, 3] who suggested its possible use in epilepsy. Subsequently, Goodman and associates[4, 5] confirmed and extended the experimental work. Thorne[6] found this drug effective against grand mal, and Richards and Perlstein[7] were the first to report its particular usefulness in petit mal. Lennox[8, 9] has reported extensive clinical studies with tridione.

Clinical experience thus far indicates that tridione is an important addition in the treatment of the petit mal triad, while its possible usefulness in other forms of epilepsy is still controversial and in need of further clarification. However, the marked effectiveness of tridione by intravenous injection is of interest in the management of status epilepticus and convulsions due to various causes. These considerations, as well as the unusual combination of both analgesic and anticonvulsant properties in one drug, justify a description of its pharmacology with special reference to therapeutic applications.

Tridione is a colorless solid with a low melting point (approximately 46° C.). It is soluble in water to 5 per cent and forms a neutral solution. In Table I are given the structural formulas of three synthetic organic drugs used in the treatment of epilepsy, namely, phenobarbital, Dilantin (diphenylhydantoin sodium), and tridione. While each compound is derived from a different cyclic structure, a common feature is the group —C(=O)—C(=O)—N—C—.

Table I

Tridione	Dilantin	Phenobarbital
		

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GENERAL PHARMACOLOGY

One of the outstanding properties of tridione is its low toxicity. The general symptomatology and toxicology is presented in a condensed form in Table II.

Sleep occurs only with very high doses, approaching lethal levels, unless the drug is injected intravenously. With lower doses the animals behave either normally or show various degrees of ataxia. If extremely high doses are given, death occurs as a result of acute respiratory and circulatory depression or after one or two days in coma. A comparison of the action in different species of animals shows qualitatively similar effects and also a rather uniform quantitative sensitivity to the drug. The lethal dose is approximately 1.5 to 2.0 Gm. per kilogram by intravenous, intraperitoneal, or subcutaneous administration, and there is also relatively little difference in the amounts necessary to induce ataxia by various routes of administration. An ataxia-producing dose given orally to mice shows its effects in two or three minutes; tying of the pylorus delays the action for about twenty-five minutes, but the drug is slowly absorbed from the stomach. Only intravenous injection produces short sleep at a dose level which was not soporific by other routes. These observations suggest that tridione is readily absorbed.

Rats fed a diet containing 0.25 per cent tridione showed no effect upon their growth rate, while concentrations of 0.5 or 1.0 per cent retarded weight gain by 15 and 40 per cent, respectively. These feeding experiments were continued for more than nine months. The red blood count of the group fed the highest concentration of the drug was found to be at the lower limit of

TABLE II. EFFECT OF TRIDIONE IN EXPERIMENTAL ANIMALS

GM./ KG.	MICE			RATS	RABBITS		CATS	DOGS
	I.V.	I.P.	ORAL	S.C.	I.V.	I.P.	I.P.	I.V.
0.25		No effect		Slight ataxia		Slight ataxia		
0.30							Slight ataxia	
0.35					Sedation	Slight ataxia		
0.40								Ataxia
0.50	Slight ataxia	Slight ataxia	Slight ataxia	Ataxia	Sedation	Ataxia		
0.60								Sleep (2 min.)
0.75	Ataxia	Ataxia	Ataxia		Sleep	Ataxia		
1.00	Light sleep	Marked ataxia	Light sleep	Marked ataxia		Marked ataxia	Sedation	Sleep (15 min.)
1.50	Sleep	Sleep	Sleep	Sleep (4 to 6 hr.)	LD_{50}	LD_{50}		
2.00	Fatal LD_{20}	Sleep	Sleep and death	LD_{50}			Prolonged sleep and death	
3.00	Acute death							

normal, while in the other groups no indication of an effect upon the hematopoietic system was noted. Urinalyses disclosed no abnormalities. Histologic sections of organs of animals that died or were killed showed some cloudy swelling of the kidney and liver and occasionally some fat in the latter organ. None of the findings was indicative of any serious organ damage.

The effect upon blood pressure and respiration was studied in dogs and cats under pentobarbital anesthesia. The rapid intravenous injection of 25 mg. of tridione per kilogram produced no effect, but 50 mg. per kilogram produced transitory depression of the blood pressure (30 to 50 mm. Hg). No significant change in respiration was seen. Etherized rabbits tolerated the slow intravenous injection of as much as 50 mg. per kilogram without respiratory depression, while 75 mg. per kilogram produced a marked depression of blood pressure and respiration. Both functions, however, gradually returned to normal.

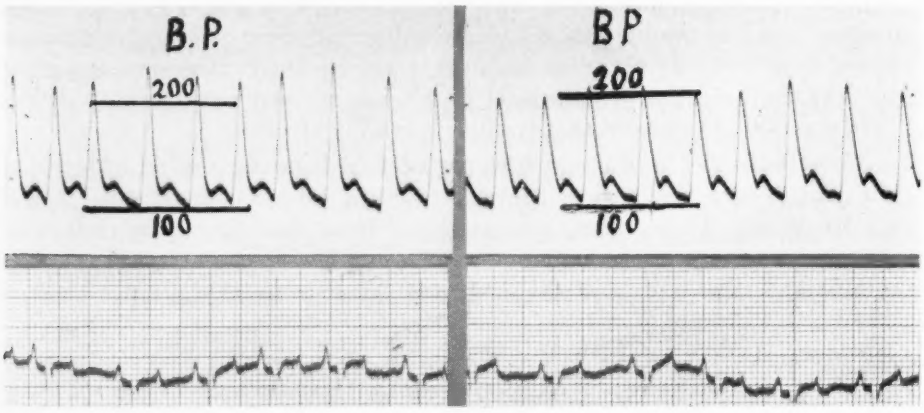

Fig. 1.—Intravenous injection of tridione in a dog weighing 16 kilograms. Upper tracings, blood pressure; lower tracing, ECG Lead II. Left before and right after a total of 3 Gm. of tridione. See text.

The action on the cardiovascular system in unanesthetized dogs was studied by simultaneous recording of the blood pressure and electrocardiograph. Blood pressure was recorded by means of a Hamilton membrane manometer. In Fig. 1 are shown tracings of a dog weighing 16 kilograms taken before the administration of the drug and after three intravenous injections, each of 1.0 Gm. of tridione given at intervals of about four minutes. Two minutes after the last dose the systolic blood pressure was transitorily reduced by approximately 15 mm., while the diastolic reading remained unchanged. No significant change in pulse rate, electrocardiogram, or respiration occurred. Observation failed to detect any sedation, and there was but slight ataxia of the hind legs. Other studies on the blood pressure and on the intestine failed to show any specific effect of tridione upon the reaction to parasympathetic stimulation by acetylcholine. Nor did the drug exhibit any significant antispasmodic action. Effects upon temperature regulation and metabolism have not yet been adequately studied.

The analgesic action of tridione was first reported by Richards and Everett,[2] and a more detailed account of the findings in experimental animals together with clinical data was given by Richards, Everett, and Pickrell.[10] The question of its practical usefulness in the clinic has not yet been settled. Certain side effects which will be discussed later appear to limit its administration as a routine analgesic to the ambulatory patient. Scrutiny of available reports and our own experience indicate that further study of the use of this drug, particularly in dysmenorrhea and for postoperative pain, is worth while, especially in view of the absence of undesirable effects upon blood pressure, respiration, and the digestive tract.

ANTICONVULSANT ACTION

The anticonvulsive action of tridione in experimental animals has been demonstrated against pentamethylenetetrazol (Metrazol) and other convulsant drugs.[3] Recently we have made use of the observation by Walker and coworkers[11] that intracortical administration of penicillin produces violent and prolonged convulsions. We found tridione effective in suppressing these seizures in rabbits and dogs. Goodman and associates[12] have reported carefully controlled experiments in which they investigated the effect of tridione and other anticonvulsant drugs upon electrically induced convulsions. Tridione was found to modify and decrease the response to electric shock and had a moderate effect upon the electroshock threshold. It was particularly effective against metrazol convulsions. Of special interest is the observation that "spike and dome" waves resembling a petit mal electroencephalogram can be produced experimentally in rabbits by subconvulsant intravenous doses of metrazol. Tridione was found to be the most effective drug in inhibiting this phenomenon; phenobarbital was less active, and dilantin was ineffective.

The duration of the anticonvulsant action was studied in rabbits. The intravenous injection of 17.5 mg. of metrazol per kilogram produced violent convulsions in each of sixteen animals so treated. If 100 mg. of tridione per kilogram were injected intravenously, ten minutes prior to the metrazol, only 30 per cent of the rabbits convulsed. In another series a forty-five minute interval was allowed between the administration of the tridione and the metrazol; convulsions occurred in 80 per cent of the animals. With a dose of 200 mg. of tridione per kilogram, only 20 per cent of the animals had convulsions when metrazol was given one hour later. These findings are in fairly good agreement with results in mice reported earlier by us.[3] It must be kept in mind that the dose of the convulsant agent was very high. Goodman and associates[12] found tridione to be protective against three to five times the convulsant dose of metrazol, depending on the species of animal. Goodman and Manuel[4] noted a residual anticonvulsive effect against the standard convulsive dose of metrazol, forty-eight hours after the intraperitoneal injection of high doses of tridione.

Information on the rate of urinary excretion of tridione was obtained in three adult men, each of whom took 2.0 Gm. of the drug in a single dose. Urine was collected at two-hour intervals during an eight-hour period. The

urine was extracted with ether, and the residue obtained after evaporation of the ether fraction was titrated with NaOH. The total amount of the drug recovered after eight hours was 47.2, 53.4, and 46.7 mg., respectively. The greater part was excreted within two to six hours after ingestion. This finding shows that only traces of therapeutic doses appear in the urine, and we must assume that the major portion of this drug is metabolized.

The role of the kidneys and liver in the metabolism of tridione was studied in nephrectomized animals and also after liver function was damaged by injection of carbon tetrachloride. If mice subjected to one or the other of these procedures were given 500 mg. of tridione per kilogram orally, no prolonged protection against the convulsive effect of 125 mg. of metrazol per kilogram was noticed in animals with damaged livers and only a slight prolongation was noticed in the nephrectomized mice. From a similar experiment in rats, it was observed that the hypnotic effect of a sublethal dose was prolonged in nephrectomized animals for twenty-four to forty-eight hours. Ataxic dosages gave more variable results but also indicated a slower disappearance of the drug. These observations indicate that therapeutic doses can apparently be destroyed in the body, even in the presence of impairment of the functions of the liver and kidney. With large doses of the drug, however, the importance of these organs becomes more prominent. Further studies are necessary to assay quantitatively the role of liver and kidneys.

As mentioned previously, the clinical usefulness of tridione in the treatment of petit mal epilepsy has been established by Richards and Perlstein,[7] by Lennox,[8, 9] and by Perlstein and Andelman,[14] and many other investigators are in general agreement. DeJong[15] found tridione effective in combination with other drugs in the treatment of psychomotor attacks. While favorable results in this disorder were also observed by Perlstein and Andelman,[14] more extensive investigation in this condition is clearly indicated. The same applies to its application in the management of disturbances of behavior in children. The need for further study with respect to the usefulness of tridione against grand mal epilepsy has been pointed out. Lennox[9] indicated that in certain patients suffering from grand and petit mal epilepsy tridione tends to increase grand mal attacks. Perlstein and Andelman[14] prefer a combination with phenobarbital, rather than dilantin, for such patients. Dramatic results in the treatment of a few cases of status epilepticus by intravenous injection of tridione have been reported by Thorne[6] and by Erickson and Masten.[16] The anticonvulsant action in patients with tetanus is reported by Perlstein and Andelman.[14]

The effect of tridione upon the electroencephalogram in petit mal does not appear to be an immediate one in all instances. Gibbs[17] has observed that several hours elapsed before an effect upon the electroencephalogram in certain cases is noted, even after intravenous administration.

SIDE EFFECTS

Minor side effects, such as drowsiness, nausea, and slight skin rashes, have been reported. Of interest is the attempt to overcome the drowsiness which

occurs in certain patients with large doses of tridione by the simultaneous administration of desoxyephedrine, a central stimulant with an action similar to that of amphetamine. Further experiments in mice have shown that it is possible to reduce the sedative effect of tridione with desoxyephedrine without significantly interfering with the protection against convulsive doses of metrazol.

Serious skin rashes in symptomatology and course, resembling those occurring after barbiturates and other drugs, have been reported in a few cases. One of us (R. K. R.) observed an adult male patient who had taken phenobarbital for a considerable time for the treatment of grand mal attacks. About ten days after tridione had been added to control petit mal seizures, the patient developed conjunctivitis quickly followed by a maculopapular rash over the whole body and by a high fever. The exanthema was particularly pronounced on the flexor, palmar, and plantar surfaces of the limbs and became exfoliative in certain areas. Macerations on the mucous membrane of the lips and mouth were present. Treatment consisted of withdrawal of both phenobarbital and tridione and administration of intravenous sodium thiosulfate and intramuscular penicillin. The fever abated after about one week, the rash faded slowly, and the patient recovered. Fortunately, severe reactions of this type are rare.

Recently two cases of blood dyscrasias terminating fatally have been reported.[18, 19] Both presented aplastic anemia and agranulocytosis. In one of these patients another new drug with potentially toxic action was administered simultaneously with tridione. In spite of the rare occurrence of such reactions, frequent blood counts should be made on all patients receiving tridione.

A peculiar side effect of tridione which we encountered early in the clinical study of this drug is the occurrence of an increased sensitivity to light. The subjective description of this sensation is referred to by some patients as "glare" and by others as a more or less marked loss of color vision which sometimes makes the objects appear yellow or covered with snow. This phenomenon is noticed particularly in bright daylight. Adults are more likely to experience this condition than children. Various degrees of visual disturbances occur in up to 75 per cent of adults in some clinical studies. This side effect appears usually within the first few weeks of the medication. Wearing of dark glasses helps considerably, and in some patients this phenomenon becomes less pronounced during continued medication. Withdrawal of the drug because of this side effect has been found necessary only in 10 to 20 per cent of adult patients (almost never in children) and was always followed by a return of normal color perception and light sensitivity. Sections of the optic nerve and retina of rats fed high doses of tridione for several weeks failed to show any organic damage. In accord with this are the negative findings of ophthalmologists on patients with marked visual symptoms. Similar symptoms sometimes occur with other drugs, for example, digitalis (Carroll[20]).

CONCLUSIONS

From the experiences of the clinical investigators, it appears that tridione will be an effective drug for the majority of patients suffering from petit mal epilepsy. Children are most benefited, since they show the greatest percentage of favorable responses with a minimum of side effects.

In this early stage in the investigation of tridione, it is not possible to make statements regarding its site of action. The origin of petit mal seizures is still obscure, but it is possible that they arise from subcortical centers. Considering the analgesic action of tridione and the role of the thalamus in pain sensation, one is induced to speculate regarding an at least partially subcortical action of tridione. It appears quite likely that further study of the drug may make it a useful tool for research in various neurophysiologic and pathologic problems.

Tridione is now being investigated clinically in various nervous disorders unrelated to epilepsy, but no statements in this respect can be made as yet.

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ACUTE AND CHRONIC TOXICITY STUDIES ON THEOPHYLLINE AMINOISOBUTANOL AND THEOPHYLLINE ETHYLENEDIAMINE

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THEOPHYLLINE derivatives have been used clinically for a number of years with favorable results.[1-4] In spite of this, the literature contains relatively few data on acute and chronic toxicity in animals. Ludueña[5] found that the repeated administration of doses equivalent to those used clinically produced no systemic effects in rabbits. He reported, however, that a single dose of theophylline ethylenediamine, approximately thirty times the clinical dose on a basis of body weight, produced irritation in the meninges and kidneys of rabbits.

Since Ludueña has reported this irritant action for one theophylline derivative, it is important that possible toxic effects of any new theophylline derivative be determined before the substance is tried clinically.

It is the purpose of this paper to present results of toxicity studies on two theophylline derivatives, theophylline aminoisobutanol and theophylline ethylenediamine which contain approximately 67 per cent and 75 to 82 per cent, respectively, of theophylline. Theophylline aminoisobutanol has recently been introduced into clinical medicine[6-8] under the trade name of Butaphyllamine, and its pharmacologic actions have been summarized by Hansel.[9] Data on theophylline ethylenediamine (Aminophylline) are included for comparative purposes and because Ludueña's report makes information on effects of prolonged administration in several species desirable.

Acute Toxicity.—The acute intravenous and oral toxicities were determined for theophylline aminoisobutanol and theophylline ethylenediamine. Intravenous toxicity determinations were conducted in rabbits. The drugs were injected into the marginal ear veins as 25 per cent aqueous solutions. Oral toxicities were determined in mice. The theophylline derivatives were administered, as 5 per cent solutions, directly into the stomach through a rigid tube.

Animals were observed for a period of one week following both intravenous and oral administrations, and all deaths occurring within this period were included in mortality curves. LD_{50} values and standard errors were determined by the method of Miller and Tainter.[10]

Results of the investigations in rabbits and mice indicate that the two compounds have similar acute toxicities. Death following administration of either compound was generally preceded by convulsions and usually occurred within ninety minutes, although in a few instances it was delayed for several days.

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Intravenously, the LD_{50} of theophylline aminoisobutanol was found to be 163 ± 5.65 mg. per kilogram and that of theophylline ethylenediamine 150 ± 7.65. Orally, the LD_{50} values of theophylline aminoisobutanol and theophylline ethylenediamine were 600 ± 34.7 and 540 ± 12.9 mg. per kilogram, respectively.

Chronic Toxicity.—The effects of repeated administration of theophylline aminoisobutanol and theophylline ethylenediamine were investigated in dogs and rats.

Dogs: Eighteen mongrel dogs were fed a diet of Purina Dog Chow and conditioned to the laboratory for a period of two months before starting the experiment. They were then divided into six groups of three dogs each. Two of the groups served as controls, one for intravenous work and one for oral work. One group was administered theophylline aminoisobutanol orally and another theophylline aminoisobutanol intravenously. The other two groups received theophylline ethylenediamine, one by the oral and one by the intravenous route. Both compounds were administered in doses of 20 mg. per kilogram per day, six days a week, for twelve weeks. These doses are equivalent to 1.2 Gm. per day for a man weighing 60 kilograms, and they are about two or three times the usual clinical dose. The drug solutions for parenteral use were prepared fresh daily in pyrogen-free sterile normal saline. Oral doses were administered in hard gelatin capsules.

Hematologic evaluations were conducted on experimental and control animals at weekly intervals throughout the course of the experiment. The evaluations included erythrocyte, reticulocyte, leucocyte, and differential cell counts and hemoglobin determinations. The hemoglobin determinations were made with a Fisher electrohemometer, and hematocrit determinations were conducted according to the Wintrobe technique[11] excepting that minimal amounts of a 10 per cent solution of potassium oxalate were added as an anticoagulant.

All dogs were sacrificed at the termination of drug administration. Specimens from the aorta, heart, lung, liver, kidney, spleen, adrenal, stomach, and small intestine were fixed in Bouin's fluid, and paraffin sections were prepared and stained with Delafield's hematoxylin and eosin. The brain meninges were fixed in formaldehyde, and sections were stained by Mallory's connective tissue method.

At the termination of drug administration, seventeen of the eighteen dogs were in excellent physical condition. They either gained in body weight, remained constant, or lost an insignificant amount. One animal developed a severe case of mange one week after starting the daily administration of theophylline aminoisobutanol orally. This dog had lost an equivalent of 20 per cent of its pre-experimental body weight by the end of the experiment; but considerable weight was lost during the predrug period, and its weight changes cannot be attributed to drug administration. It is concluded, therefore, that the daily administration of the two theophylline derivatives did not prove toxic, as measured by changes in body weight and general condition of the experimental animals.

The two theophylline derivatives did not alter the blood pictures of the dogs. Initial and final average values (Table I), which are typical of the

TABLE I. INITIAL AND FINAL AVERAGE BLOOD VALUES WITH STANDARD ERRORS FOR CONTROL AND EXPERIMENTAL DOGS

COMPOUND	ERYTHROCYTES (MILLIONS PER C. MM.)		HEMATOCRIT (PER CENT CELLS)		RETICULOCYTES (PER CENT)		HEMOGLOBIN (GM. PER 100 C.C.)		LEUCOCYTES			
									THOUSANDS PER C. MM.		GRANULOCYTES PER CENT	
	INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL
Intravenous Administration												
Theophylline aminoisobutanol	6.36 ± 0.01	6.20 ± 0.30	37.0 ± 3.9	36.0 ± 3.6	0	0.40 ± 0.26	12.6 ± 0.4	13.4 ± 0.4	11.7 ± 1.2	11.2 ± 0.4	70.8 ± 9.7	62.0 ± 6.5
Theophylline ethylenediamine	6.35 ± 0.25	6.72 ± 0.51	34.3 ± 1.9	38.0 ± 1.7	0	0.03 ± 0.01	12.4 ± 0.1	13.5 ± 0.6	10.9 ± 0.8	11.4 ± 0.2	69.0 ± 2.5	63.2 ± 8.7
Control	5.49 ± 0.16	6.24 ± 0.15	38.6 ± 1.5	36.0 ± 2.4	0.33 ± 0.24	0.23 ± 0.23	11.7 ± 0.9	13.3 ± 0.9	12.2 ± 2.7	12.6 ± 2.3	67.0 ± 3.6	68.7 ± 6.7
Oral Administration												
Theophylline aminoisobutanol	6.26 ± 0.48	6.98 ± 0.51	37.0 ± 0.6	39.0 ± 1.2	0	0.17 ± 0.17	12.1 ± 0.4	14.3 ± 0.5	15.1 ± 0.2	10.4 ± 0.9	64.0 ± 11.7	76.0 ± 3.0
Theophylline ethylenediamine	6.75 ± 0.55	7.50 ± 0.36	40.0 ± 2.2	39.0 ± 3.0	0.03 ± 0.01	0.20 ± 0.20	13.4 ± 0.8	14.8 ± 1.2	9.7 ± 0.3	10.0 ± 1.0	69.8 ± 3.6	71.5 ± 3.2
Control	6.82 ± 0.12	6.58 ± 0.15	37.0 ± 1.3	40.0 ± 2.5	0.07 ± 0.01	0.23 ± 0.21	13.3 ± 1.3	14.6 ± 0.8	11.1 ± 1.0	12.1 ± 2.0	65.7 ± 2.0	72.3 ± 7.4

weekly values, fall within a normal range, and there is no significant difference in data for the experimental and control groups.

Microscopic examination of tissue sections from sacrificed animals revealed only minimal pathologic changes and these were not significant. Some abnormalities were found in both control and experimental animals, and the changes were of a type frequently encountered in laboratory animals. They included pneumonia in one dog, thinning of the cytoplasm of liver cells in one,

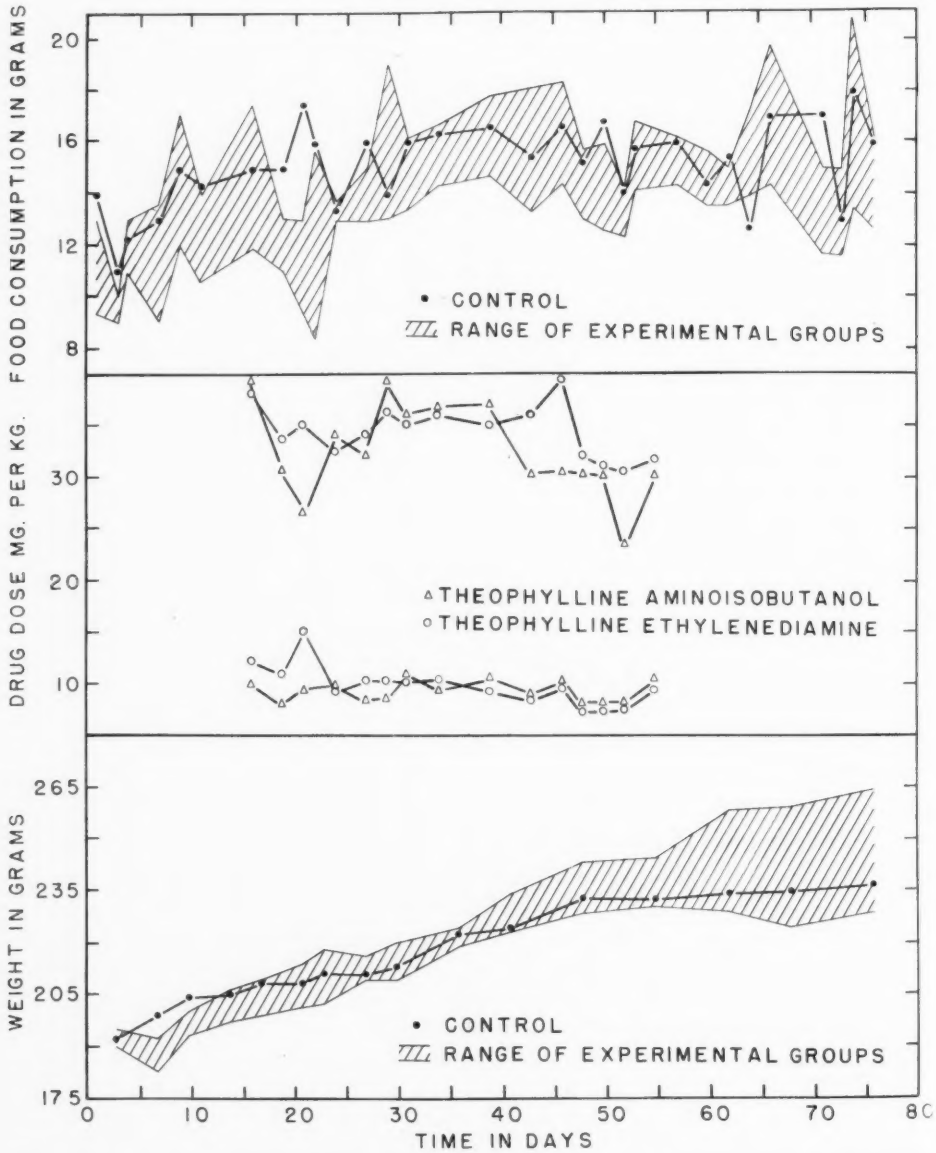

Fig. 1.—Average food consumption, drug dose, and body weight for experimental and control female rats.

TABLE II. INITIAL AND FINAL AVERAGE BLOOD VALUES WITH STANDARD ERRORS FOR CONTROL AND EXPERIMENTAL RATS

COMPOUND	DOSE (MG. PER KG.)	ERYTHROCYTES (MILLIONS PER C. MM.)		RETICULOCYTES (PER CENT)		HEMOGLOBIN (GM. PER 100 C.C.)		LEUCOCYTES			
								THOUSANDS PER C. MM.		GRANULOCYTES PER CENT	
		INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL
Female Rats											
Theophylline aminoisobutanol	9	7.83 ± 0.41	8.19 ± 0.33	1.9 ± 0.6	1.2 ± 0.2	15.5 ± 1.1	14.3 ± 0.4	16.4 ± 2.4	15.4 ± 2.0	25.4 ± 3.4	28.4 ± 3.8
	34	7.74 ± 0.26	8.62 ± 0.14	2.0 ± 0.3	1.9 ± 0.3	13.3 ± 0.3	14.7 ± 0.6	12.5 ± 0.2	13.3 ± 0.7	21.1 ± 3.3	26.5 ± 3.3
Theophylline ethylenediamine	9	8.30 ± 0.17	8.52 ± 0.13	1.1 ± 0.9	1.4 ± 0.3	14.3 ± 0.7	14.4 ± 0.5	18.9 ± 3.0	15.7 ± 3.4	19.0 ± 1.5	25.1 ± 2.7
	34	7.68 ± 0.30	8.14 ± 0.38	1.7 ± 0.4	1.0 ± 0.1	13.6 ± 0.2	14.0 ± 0.4	16.4 ± 1.6	17.0 ± 1.0	19.6 ± 3.1	26.0 ± 3.6
Control	--	8.11 ± 0.23	8.03 ± 0.36	3.0 ± 0.9	1.9 ± 0.2	14.1 ± 0.4	13.3 ± 0.4	15.7 ± 1.9	16.0 ± 2.1	17.4 ± 2.0	26.0 ± 4.6
Male Rats											
Theophylline aminoisobutanol	9	7.83 ± 0.29	9.35 ± 0.35	3.5 ± 0.3	2.0 ± 0.3	11.6 ± 0.7	13.9 ± 0.6	17.5 ± 1.5	18.5 ± 0.8	26.1 ± 3.1	29.4 ± 2.3
	34	8.41 ± 0.33	9.60 ± 0.22	2.1 ± 0.4	1.9 ± 0.5	14.3 ± 0.6	14.7 ± 0.7	15.7 ± 0.7	21.6 ± 2.8	18.1 ± 1.1	25.8 ± 2.8
Theophylline ethylenediamine	9	7.70 ± 0.34	9.28 ± 0.07	4.3 ± 1.5	2.6 ± 0.5	12.5 ± 0.8	14.2 ± 0.8	20.0 ± 2.7	19.7 ± 2.2	22.4 ± 1.7	21.0 ± 2.6
	34	7.94 ± 0.33	9.02 ± 0.25	3.2 ± 0.9	2.7 ± 1.8	13.0 ± 0.4	13.3 ± 0.5	18.4 ± 2.3	22.9 ± 0.3	16.3 ± 1.5	27.3 ± 4.6
Control	--	8.57 ± 0.31	9.80 ± 0.15	2.3 ± 0.4	2.1 ± 0.4	13.9 ± 0.5	15.0 ± 0.5	18.8 ± 1.9	17.8 ± 2.1	19.5 ± 1.6	22.6 ± 5.2

nuclear pyknosis in a few scattered parenchymatous liver cells in two, and minimal amounts of nuclear pyknosis in cells of the nephronic tubules in two animals.

Rats: Five groups of six male and five groups of six female rats of the Wistar strain were employed in evaluating effects of repeated administration of the two derivatives to rats. One group of male and one group of female animals served as controls. The experimental groups received either theophylline aminoisobutanol or theophylline ethylenediamine in doses of approximately 9 and 34 mg., respectively, per kilogram per day by the drug-diet method.

Drug administration was continued for forty days in the female and sixty days in the male animals. One-half of the rats from each group were sacrificed at the termination of drug administration, and the remaining animals were sacrificed three weeks later. Tissues from all experimental and control animals were fixed in Bouin's fluid, and paraffin sections of the aorta, heart, lung, liver, kidney, spleen, adrenal, stomach, and small and large intestines were prepared.

Data in Fig. 1 indicate that the two theophylline derivatives did not alter food consumption or body weight of female animals. Similar results were obtained with male animals; therefore, data for these rats are not presented.

Neither compound caused blood dyscrasia. Erythrocyte, reticulocyte, total and differential leucocyte counts, and hemoglobin concentrations remained in the normal range (Table II), and values for experimental groups did not differ significantly from those of controls.

Microscopic examination of tissue sections revealed no pathologic changes which could be attributed to the theophylline derivatives, either in animals sacrificed at the termination of drug administration or in animals sacrificed three weeks later. The histologic alterations observed were of types commonly found in rats, and they occurred as frequently in control as in experimental groups.

SUMMARY

Theophylline aminoisobutanol and theophylline ethylenediamine were found to have similar LD_{50} values for the oral route in mice and the intravenous route in rabbits, and both compounds produced similar signs of acute toxicity.

The two compounds produced no evidence of toxicity in dogs following the daily administration of 20 mg. per kilogram, either intravenously or orally, and no toxic effects were observed in rats fed amounts of the drugs up to 34 mg. per kilogram daily for as long as sixty days.

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STUDIES ON THE PLASMA CONCENTRATION AND TISSUE DISTRIBUTION OF SODIUM PENTOTHAL (SODIUM ETHYL (1-METHYLBUTYL) THIOBARBITURATE)

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INTRODUCTION

IN RECENT years sodium pentothal* has become important in anesthesia, especially in the Armed Forces. However, very little is known about its physiologic distribution in the body and its concentration in biologic fluids. The greatest difficulty has been the lack of a specific method for the determination of the drug.

The method commonly employed in the determination of various barbiturates has been the Koppanyi reaction[1] and its modifications.[2,3] This is a colorimetric method based on the reaction between a barbiturate, cobalt acetate, and isopropyl amine. The reaction is admittedly nonspecific and lacks the sensitivity and accuracy needed for repeated blood level measurements on the same animal.

Anderson and Essex,[4, 5] employing the Koppanyi reaction, studied the plasma levels of pentothal under various conditions and claimed that the drug underwent a cyclic appearance and disappearance in the blood, and there was no correlation between the depth of anesthesia and the blood concentration. This is an unusual phenomenon, for rarely does a drug act in such a cyclic manner.

The purpose of this study was to determine the behavior of pentothal in the blood and tissues after intravenous administration and, if possible, to correlate the depth of anesthesia with the blood level. For such a study a new method of determination was devised which is more specific and more accurate than the Koppanyi reaction.

METHODS AND MATERIALS

Hellman and associates[6] and Brodie[7] have studied the ultraviolet absorption curves of pentothal in both organic and aqueous solvents. This is the basis for the present method of determination.

Procedure.—Plasma (0.5 to 1.0 c.c.) and 0.2 M secondary sodium phosphate and 0.1 M citric acid buffer of pH 5 (2 c.c.) are added to redistilled chloroform (7 c.c.) in a small separatory funnel and shaken for three minutes. The two phases are allowed to separate and the chloroform layer is filtered to remove the water droplets. This clear chloroform solution is read in a Beckman quartz

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*Use of the chemical name, sodium ethyl (1-methylbutyl) thiobarbiturate, would be so cumbersome that use of the proprietary designation has been permitted throughout this paper.

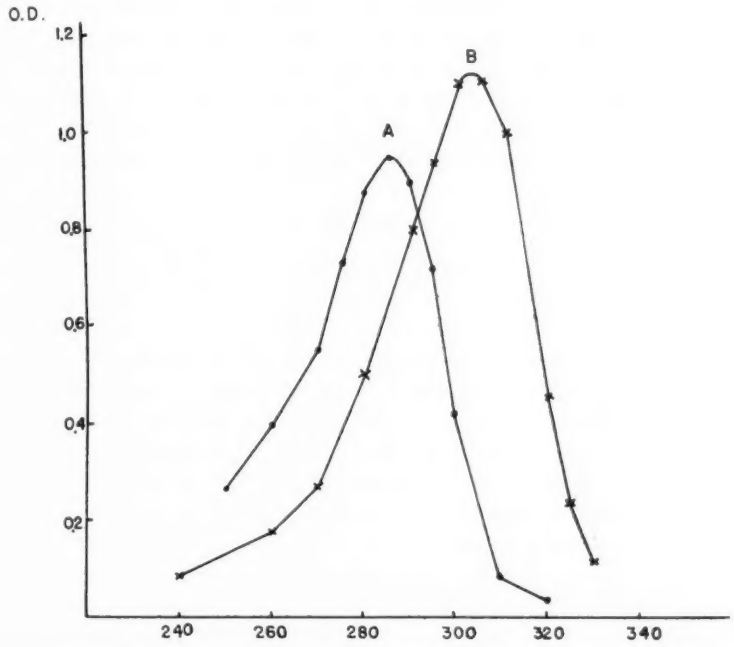

Fig. 1.—Absorption curves of pentothal (*A*) and sodium pentothal (*B*) in chloroform and 0.2 N NaOH, respectively. The ordinate represents optical density (2 -log. G) and the abscissa millimicrons.

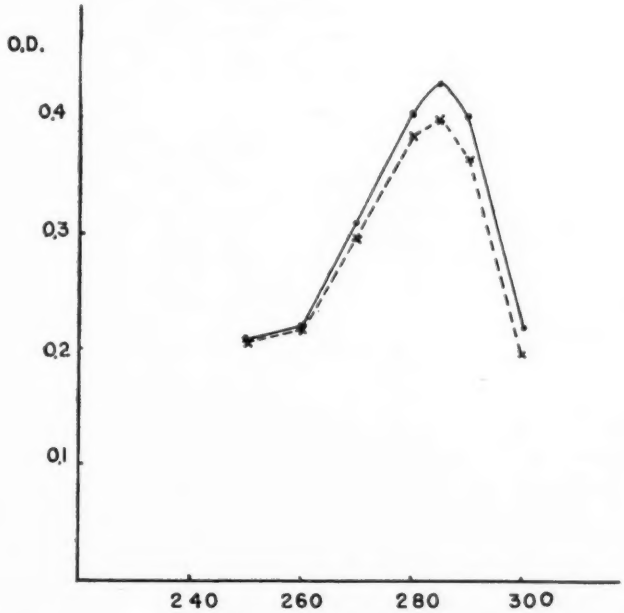

Fig. 2.—Comparison of absorption curves of chloroform extract of rabbit plasma to which pentothal had been added (solid line) and of a similar extract of plasma from a rabbit given sodium pentothal intravenously (broken line).

spectrophotometer at 285 millimicrons. If the chloroform layer is extracted with 0.2 N NaOH and the latter read at 305 mμ, the sensitivity is somewhat increased. The order of magnitude of sensitivity is 0.5 μg per cubic centimeter of the final solution of chloroform or NaOH. Pentothal was prepared by acidifying pentothal sodium and extracting with ether. This was further purified by sublimation under reduced pressure. The resultant pure crystals were used for standards.

The absorption curve of pentothal in chloroform and in 0.2 N NaOH is presented in Fig. 1, and the maximal absorption at 285 and 305 mμ, respectively, is shown. At these wave lengths there is a linear relationship between the concentration and the optical density. Experiments were performed with known amounts of pentothal added to plasma, whole blood, and tissue homogenates, and the recoveries averaged over 90 per cent. In all experiments control plasma was used as a blank (which is small), and known amounts of drug were added to the control in order to determine the percentage recovery (Table I). The specificity of the method is shown in Fig. 2, where the absorption curve of pentothal added to control plasma and extracted with chloroform is compared with that extracted from the plasma of a rabbit which had received the drug intravenously. It is apparent that the materials extracted are identical spectrophotometrically.

In the pharmacologic experiments, five rabbits were slowly injected intravenously with 50 to 100 mg. of sodium pentothal, and blood samples were taken at various intervals by cardiac puncture. In the study of the physiologic disposition of the drug, three rabbits were given 150 mg. and were killed by an air embolus twenty minutes later. The tissue was homogenized in phosphate buffer of pH 5 (0.2 M secondary sodium phosphate and 0.1 M citric

TABLE I

SUBSTANCE	PENTOTHAL ADDED (μG)	PENTOTHAL RECOVERED (μG)	PERCENTAGE RECOVERY
Plasma (c.c.)			
1.0	12.5	12.0	95
1.0	20.0	22.0	108
1.0	20.0	19.0	95
1.0	25.0	23.0	92
Whole Blood (c.c.)			
1.0	10.0	9.9	99
1.0	20.0	19.0	95
1.0	20.0	19.5	98
Liver (Gm.)			
0.5	60.0	58.0	95
1.0	20.0	19.4	96
1.0	20.0	19.6	98
1.5	60.0	56.0	92
Kidney (Gm.)			
1.0	20.0	18.5	92
1.0	20.0	19.0	95
Muscle (Gm.)			
1.0	20.0	19.0	95
1.0	20.0	18.5	92
Brain (Gm.)			
0.5	20.0	19.0	95
1.0	20.0	18.0	90

acid), and the procedure for plasma previously described was followed. Two patients at Walter Reed General Hospital, with only 0.3 mg. of atropine sulfate as preoperative medication, were given pentothal in the usual manner by intermittent intravenous injection in the arm. Blood for plasma levels was taken from the other arm.*

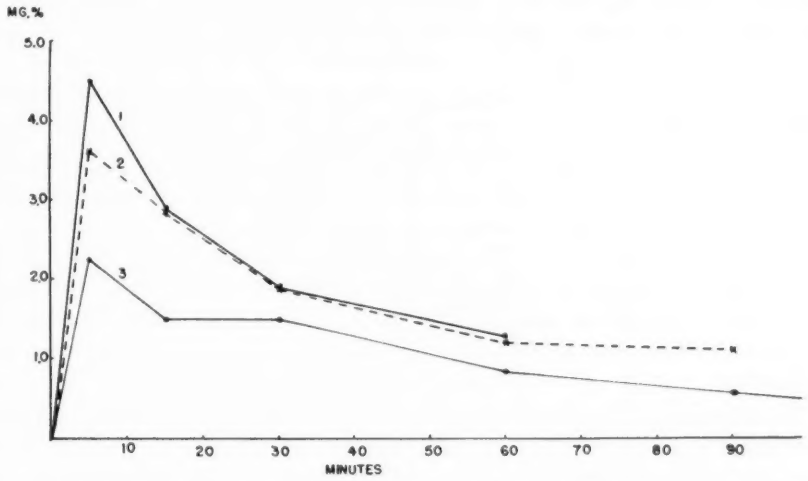

Fig. 3.—Plasma concentration after administration of sodium pentothal intravenously. Rabbits 1 and 2 received 100 mg.; Rabbit 3, 50 milligrams.

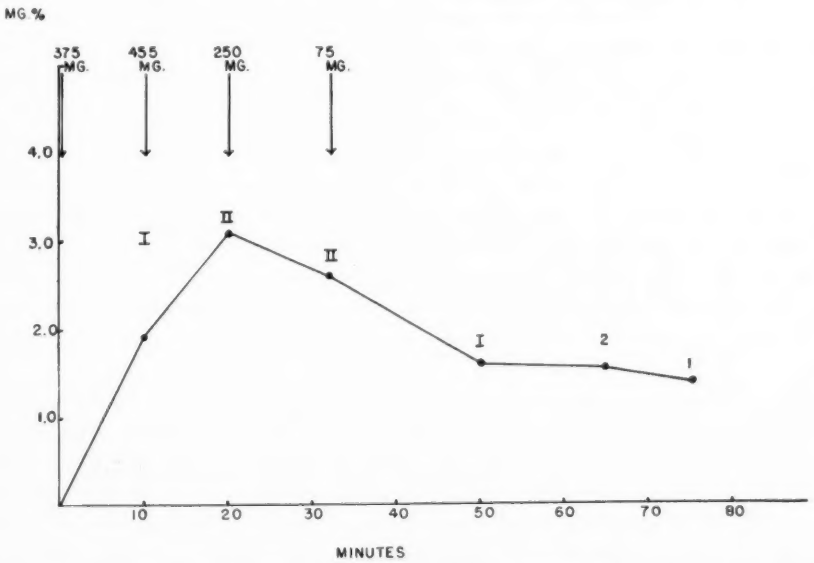

Fig. 4.—Plasma concentrations in a human patient during and after administration of sodium pentothal. The arrows show the beginning of the intermittent injections of the indicated amount of pentothal. Those blood samples taken at 10, 20, 32, 50, 65, and 73 minutes following the first dose of drug were withdrawn just prior to administration of the second, third, and fourth doses, respectively. "I" and "II" refer to the plane of surgical anesthesia; "1" and "2" refer to the stage of anesthesia.

*We wish to express our appreciation to Major Emanuel Papper, Chief of the Department of Anesthesia, for doing the clinical aspects of this experiment.

RESULTS

In Fig. 3 is illustrated the course of the plasma level of pentothal after intravenous injection of sodium pentothal in rabbits. The plasma level reaches a peak and falls off rapidly. There is no evidence of any cyclic rise and fall. There seems to be a correlation between the plasma level and the depth of anesthesia. As the anesthesia wears off the plasma level falls. In all cases the concentration of the drug in the plasma was below 1.5 mg. per 100 c.c. when the animal was awake.

Since it is difficult to ascertain the depth of anesthesia in the rabbit, two patients undergoing surgery with pentothal as an anesthetic were studied. In Fig. 4 the stage of anesthesia with the plasma level is correlated. This is merely a preliminary study and more clinical studies are necessary to establish a relationship between concentration and action.

Physiologic Disposition.—The amount of pentothal found in the urine of both rabbits and patients after the administration of pentothal is negligible. Thus, urinary excretion plays a very minor role in the rapid decline of the plasma level. For example, after 1.5 Gm. were given to a patient, only 1.4 mg. were recovered in the urine within a period of two hours.

Whole blood contains approximately the same concentration of pentothal as the plasma, so that it appears that there is no storage of the drug in either the red or the white cells.

TABLE II. TISSUE LEVELS TWENTY MINUTES AFTER INTRAVENOUS ADMINISTRATION OF 150 MG. OF SODIUM PENTOTHAL IN RABBITS WEIGHING 3 KILOGRAMS EACH (VALUES IN MILLIGRAMS PER 100 C.C. OR 100 GM.)

NO.	PLASMA	WHOLE BLOOD	LIVER	KIDNEY	BRAIN	MUSCLE
1	4.1	3.7	6.9	8.4	2.1	1.9
2	4.6		6.9	12.0	3.1	2.4
3	2.6	2.4	4.3	9.4	1.3	1.0

The distribution of the drug, twenty minutes after the intravenous administration of 150 mg. to a rabbit, is illustrated in Table II. The drug is not stored to any appreciable degree. The kidneys contain more than any other organ, in spite of the fact that so little is excreted in the urine.

DISCUSSION

Ultraviolet spectrophotometry offers a simple, sensitive, specific, and accurate method for the estimation of pentothal in biologic fluids. The substance extracted from the blood after the administration of the drug is identical spectrophotometrically with the crystalline material. Another promising method for the determination of pentothal depends on its fluorescence in strongly alkaline solutions. However, the emitted light has too short a wave length for great sensitivity with the filters at our disposal.

From the data presented, it appears that after the intravenous administration of pentothal the plasma concentration reaches a peak in a short time and falls off rapidly. This decrease is similar to that found with many other drugs, for example, sulfonamides and atabrine. There is no evidence of any

cyclic behavior of disappearance and appearance of the drug in the blood, as has been claimed. There appears to be some correlation between the depth of anesthesia and the concentration of drug in the plasma. Further study of clinical material is necessary to permit an accurate expression of this relationship.

Sodium pentothal is a short-acting barbiturate. Its short action is accompanied by a rapid fall in its plasma concentration after the cessation of the injection of the drug. Negligible amounts are excreted in the urine, and there is very little storage of the drug in the organs of the body. For example, by calculation from Table II twenty minutes after the administration of the drug, only about 20 per cent of the drug administered can be accounted for in the tissues. Therefore, degradation must proceed very rapidly.

SUMMARY

1. A spectrophotometric method for the determination of pentothal has been devised which is simple, accurate, sensitive, and specific.

2. The plasma concentration quickly reaches a maximum level soon after intravenous administration and then falls off rapidly. There seems to be a correlation between the depth of anesthesia and the plasma level.

3. Pentothal is degraded rapidly in the body, since very little is excreted in the urine and there is but a small amount of storage in the organs.

We wish to express our appreciation to Dr. S. G. Hershey for suggesting this problem.

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PYRIBENZAMINE IN HAY FEVER AND OTHER ALLERGIC DISORDERS

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PYRIBENZAMINE is one of a new group of synthetic substances which exert a palliative action on certain allergic manifestations. Experimentally these compounds possess the ability of antagonizing the pharmacologic effects of histamine, which is thought to play some role in the allergic reaction. In comparison to other related compounds the activity of Pyribenzamine is relatively high. Anaphylaxis in laboratory animals is also modified and in some instances prevented by the prior administration of this drug.[1-5]

Pyribenzamine* was administered to over 200 patients manifesting one or more allergic disorders. The drug was supplied by the manufacturer in the form of 50 mg. scored tablets for oral administration. The usual dosage in adults consisted of 50 to 100 mg. four times daily, after meals and at bedtime. In children, 25 to 50 mg. doses were usually employed. Drowsiness, nausea, and vertigo were the most frequent side effects and limited the administration of the drug in many instances.

POLLINOSIS

The effect of pyribenzamine in ninety-eight cases of hay fever was studied. Twenty-eight were due to grass pollen and the remainder to ragweed. The majority of patients had no desensitization treatment to the specific pollen or had received so little that effective results could not be expected from that form of therapy. Some had received adequate pollen treatment but still had symptoms which required attention. For the most part, patients in this group who received no previous desensitization treatment had the severest symptoms.

The evaluation of any therapeutic measure in hay fever must take into account the variation of the daily pollen concentration of the air as well as changing atmospheric conditions. These factors frequently account for increase and decrease in symptoms which can erroneously be attributed to the treatment being administered at those particular times. In addition, patients vary widely in their response to similar amounts of pollen exposure. Some will have minimal symptoms consisting of sniffling and an occasional sneeze, while others will have very marked symptoms such as rhinitis, nasal blocking, episodes of sneezing and conjunctivitis, and irritation of the throat, one or all of which may be present throughout the twenty-four-hour period. The symptoms of many lie between these two extremes. We have classified patients into minimal, mild, and severe, according to the severity of symptoms, in order to better evaluate the effect of pyribenzamine in hay fever.

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*The Pyribenzamine was supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

Where the action of the drug was favorable, its effect usually became apparent within thirty minutes and lasted several hours. Rhinorrhea and sneezing were more readily alleviated than nasal blocking.

In twenty-eight patients with grass pollinosis (Table I), nineteen received symptomatic relief, while nine showed no satisfactory change. Six of these patients were considered to have had severe symptoms, while twenty-two had mild or minimal difficulty. An appreciable amount of benefit was obtained in the majority of patients in each group.

TABLE I. GRASS HAY FEVER

	MINIMAL SYMPTOMS	MILD SYMPTOMS	SEVERE SYMPTOMS	TOTAL
Improved	8	7	4	19
Not improved	1	6	2	9
Total	9	13	6	28

TABLE II. RAGWEED HAY FEVER

	MINIMAL SYMPTOMS	MILD SYMPTOMS	SEVERE SYMPTOMS	TOTAL
Improved on high and low pollen days	18	7	1	26
Improved on low pollen days only	2	7	11	20
Not improved	5	10	9	24
Total	25	24	21	70

Seventy cases of ragweed hay fever received pyribenzamine (Table II). Attention to pollen counts and comparison with control patients were used in evaluating these results. In many cases symptoms were not completely relieved by the drug; however, where an appreciable improvement was noted, the patient was considered benefited. Twenty-one cases were classified as severe hay fever, twenty-four as mild, and twenty-five as minimal. Since it was found that in the same patient the drug frequently helped on some days, and failed to do so on others, its efficiency was further analyzed according to the atmospheric pollen concentration of that date. Of twenty-one patients with severe hay fever, eleven obtained satisfactory symptomatic relief during periods of low pollen concentration. Only one of the total group, however, obtained discernible benefit when the pollen count was high. In twenty-four patients with mild hay fever, seven were benefited during periods of both high and low pollen counts, while seven others obtained relief on low pollen days only. Eighteen of twenty-five patients with minimal symptoms obtained excellent results during periods of both high and low pollen concentrations. Two other patients in this group noted appreciable benefit only when the pollen count was low. Seven patients with seasonal asthma associated with severe hay fever were encountered during the ragweed season. Pyribenzamine was helpful in relieving asthma in one instance.

OTHER ALLERGIC CONDITIONS (TABLE III)

Nonseasonal Asthma and Allergic Rhinitis.—Thirty patients with nonseasonal asthma received pyribenzamine. Two of these received symptomatic relief whenever the drug was administered. The remainder of the patients did

TABLE III. OTHER ALLERGIC CONDITIONS

CONDITION	TOTAL CASES	HELPED	NOT HELPED
Vasomotor rhinitis	108	69	39
Asthma, nonseasonal	30	2	28
Urticaria and angioneurotic edema			
Acute	9	8	1
Chronic	15	11	4
Eczema			
Atopic	6	5	1
Contact	6	2	4
Pruritis, generalized	8	6	2
Dermatitis, unclassified	4	1	3
Headache allergic	12	0	12
Conjunctivitis, vernal	4	0	4
Blepharitis, allergic	2	1	1
Gastrointestinal allergy	2	1	1

not experience relief of asthma, although in many cases where allergic rhinitis was also present, a beneficial effect on the nasal symptoms was evident. Of 108 patients with allergic rhinitis, with and without asthma, symptoms were alleviated to some extent by the drug in sixty-nine. The degree of relief in each patient was extremely variable, and just as in seasonal hay fever the symptoms of rhinorrhea and sneezing were more amenable to control than nasal stuffiness. Pyribenzamine appears to be more effective than oral doses of ephedrine in controlling allergic nasal symptoms. In the presence of complicating nasal infection, evidenced by a purulent nasal secretion, the efficacy of pyribenzamine seems diminished. In asthma, ephedrine is still the drug of choice in most cases.

Allergic Skin Disorders.—Pyribenzamine was found very effective in controlling certain cases of urticaria and angioneurotic edema. Eleven patients with chronic urticaria were completely relieved during its administration, while four others failed to attain any appreciable benefit. It was found extremely helpful in controlling symptoms when allergic studies were being carried out in these patients to determine the specific etiologic agent. Eight of nine patients with acute urticaria obtained striking relief of symptoms following the first or second dose of pyribenzamine. The drug was found to exert a rather marked antipruritic action. This property together with the sedative effect frequently accompanying its use were of considerable value in controlling the itching incident to such allergic skin disorders as atopic dermatitis. Pruritis associated with other conditions was also frequently relieved by the drug.

Miscellaneous Allergies.—Twelve patients with headache in whom allergy was considered to play a major role were not helped by pyribenzamine. No benefit was apparent in four cases of vernal conjunctivitis. One of two cases of blepharitis was improved by its use. A patient with gastrointestinal allergy claimed relief of abdominal cramps a short time after a dose of 50 mg. was taken. Another similar case was unaffected by maximum doses.

TOXICITY

Pharmacologic studies in laboratory animals indicate that the toxicity of pyribenzamine is relatively low.[5] Side effects attending its clinical use in this study occurred in 27.2 per cent of the patients. Very often the same patient

TABLE IV. SIDE EFFECTS

SYMPTOM	NUMBER OF PATIENTS
Gastrointestinal symptoms	19
Drowsiness	18
Vertigo	16
Fatigue	9
Insomnia	6
Nervousness	4
Dryness of mucous membranes	4
Increased nasal congestion	3
Palpitation	2
Headache	2
Numbness of oral mucous membranes	1
Numbness of hands and feet	1
Feeling of tightness in chest	1
Extrasystole	1
Disorientation	1
Dysuria	1
Urticaria	1

experienced more than one undesirable symptom from the drug. The types of symptoms and the frequency with which they occurred are listed in the accompanying chart (Table IV). While gastrointestinal upsets, drowsiness, and vertigo were the most common symptoms, totally different effects such as insomnia, palpitation, and nervousness were occasionally encountered. Three patients experienced increased nasal stuffiness following the use of the drug. Another complained of asthma following each dose, and one patient, who was taking pyribenzamine for symptomatic relief in vasomotor rhinitis with good results, developed urticaria which was found definitely related to the ingestion of the drug. No evidence of chronic toxicity was observed in any of the patients treated.

DISCUSSION

The favorable effect of pyribenzamine in many cases of allergy is in support of the theory that histamine is a factor in these conditions. If histamine is considered to play the principal role in allergic reactions, the ability of pyribenzamine to more readily control certain types of allergy than others must be explained. Factors other than histamine may of course be held accountable. On the other hand, a quantitative difference in histamine release could be responsible for this variability in action. The observations made in cases of hay fever suggest such a possibility. Although approximately 66 per cent of these cases obtained some benefit from the drug, a closer analysis reveals that its effectiveness was most apparent in sufferers with less than severe symptoms, and almost half of these patients obtained relief only during days of low pollen concentration. It is possible that in severe types of hay fever, and in many cases of asthma, the histamine release is excessive and not adequately antagonized by usual doses of pyribenzamine. Larger doses might produce the desired effect in these instances. In the present study, however, the average patient tolerated doses of more than 100 mg. rather poorly.

The action of pyribenzamine is palliative and of relatively brief duration. There is as yet no proof of lasting benefit or permanent cure from its use. It therefore does not eliminate the need for a thorough allergic investigation of

the individual case and for therapy based on immunologic principles where indicated. It is a valuable adjunct to the specific treatment of hay fever, perennial allergic rhinitis, and urticaria.

SUMMARY

1. Pyribenzamine, a drug with specific antihistamine action, was used in various types of allergic disorders. It afforded symptomatic relief in many cases of seasonal hay fever, perennial allergic rhinitis, and urticaria. Its action in asthma was negligible in most instances.

2. Side effects occurred in 27.2 per cent of those patients treated. Drowsiness, gastrointestinal upsets, and vertigo were the most frequent complaints.

3. The action of the drug is only palliative and does not cure the allergic condition. It is a valuable symptomatic drug and is recommmended as a helpful adjunct to the specific treatment of allergic disorders.

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LABORATORY METHODS

STUDIES ON STREPTOMYCIN*

III. Microtechniques for Assay

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VARIOUS methods of assaying streptomycin and streptothricin described in the literature are, for the most part, modifications of penicillin assay techniques. Using a sensitive strain of *Bacillus subtilis* as a test organism for streptothricin determinations, Foster and Woodruf[1, 2] employed a modified cylinder-plate method as described by Abraham and associates[3] for penicillin determinations. Similarly, Denkelwater, Cook, and Tishler[4] employed the cup method for assay to determine the degree of inactivation of streptomycin and streptothricin by cysteine. The turbidimetric method, the serial dilution procedure with *Escherichia coli* as the test organism, and the cylinder-plate technique using *B. subtilis* cells or spores were suggested by Waksman, Bugie, and Schatz.[5] Stebbins and Robinson[6] proposed a modified agar cup-plate method for assaying streptomycin in body fluids, using a special strain of *Staphylococcus aureus* as a test organism. For estimating the concentration of streptomycin in body fluids, Heilman[7] employed a technique similar to Fleming's[8, 9] slide-cell method; *Bacillus megatherium* was used as the test organism instead of *Streptococcus pyogenes.* Fleming found it necessary to heat blood for thirty minutes at 50° C. to kill leucocytes when assaying whole blood. Donovick and co-workers[10] recommended a broth dilution method, using a sensitive strain of *Klebsiella pneumoniae* as a test organism. It was found that fluids to be assayed had to be sterile and had to be diluted to contain from 1.0 to 3.0 units of streptomycin per cubic centimeter. Price, Nielsen, and Welch[11] suggested a turbidimetric serial dilution method, using *Bacillus circulans* as the test organism, which they found sensitive to 0.15 microgram of streptomycin base per cubic centimeter of fluid. A method for the quantitative determination of streptomycin in aqueous solutions and in various organic solutions by the filter paper disk technique using *B. subtilis* as a test organism was described by Loo and associates.[12] By rigid standardization, Levy Schwed, and Sackett[13] obtained a polarographic method for assaying solutions of partly purified streptomycin containing 200 units per cubic centimeter or more of the drug. Using a gram-negative rod for a test organism and a modification of the assay method of Stebbins and Robinson,[6] Smith[14] detected as little as 0.1 per cent

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*Unless otherwise designated, streptomycin as referred to in this paper is streptomycin hydrochloride. One unit of streptomycin as used in this publication is that amount of material which will inhibit the growth of a standard strain of *Escherichia coli* in 1 c.c. of nutrient broth or other suitable medium.

of streptothricin present as a contaminant in streptomycin. While the organism was inhibited by 1,000 or more units of streptomycin, it was inhibited by only 7.5 units of streptothricin.

In a recent publication, Forgacs, Kornegay, and Henley[15] described a pad method of streptomycin assay to be used primarily for oxalated whole blood and other body fluids. Although the method proved to be accurate to 0.5 unit of the drug per cubic centimeter of body fluid, it required 20 c.c. for a complete standard curve and 1 c.c. for a blood level determination when assaying in quintuplet. This made it difficult to follow drug level determinations in infants or in individual small laboratory animals.

Two methods, described in this publication and designated as the micromethod and microfilm method, circumvent this difficulty. Using the micromethod, 1.5 c.c. of blood are required for a complete standard curve (0.5 through 40 units) and 0.06 c.c. for a drug level determination in quintuplet. For a low unitage standard curve (0.5 through 15 units), only 1 c.c. is required. When using the microfilm method of assay, 1 c.c. of body fluid is required for a standard curve (0.1 through 1.0 unit) and the same amount for a drug level determination as required by the micromethod.

EXPERIMENTAL METHODS

Preparation of Spore Suspension.—

Strain: A variant strain of *B. subtilis* Cohn *emend.* Prasmowski, designated in this laboratory as the I strain, was chosen as the standard assay organism for reasons stated in a previous publication.[15] The techniques described therein were used for preparing spores. Since difficulty was experienced by various workers in sporulating this organism when using the medium previously recommended, a further study of the medium is in progress and results will be reported at a later date. Spores used in this investigation, however, were prepared on the medium previously recommended.

Assay Technique for Micromethod.—

Preparation of Seeded Plates: For assaying, 200 c.c. of Difco E-2 Streptothricin Assay Agar were melted, cooled, and maintained at 50° C. in a water bath. Prior to assay, the agar was seeded with *B. subtilis* spores (approximately 400,000 spores per cubic centimeter of agar) and maintained at 50° C. until all air bubbles emerged to the surface. Twenty cubic centimeter portions of inoculated agar were transferred to large flat-bottomed Petri plates (150 by 20 mm.), using a 30 c.c. glass syringe equipped with an automatic syringe filler to facilitate rapid pouring. (For 100 by 10 mm. Petri plates, 10 c.c. of seeded agar were poured per plate.) Seeded plates were allowed to cool and harden for ten minutes at room temperature. They were then dried uncovered at 50° C. for ten minutes to remove condensed moisture and allowed to return to room temperature for ten minutes with covers partly open.

It was found essential that plating be performed on a level table to obtain a uniform depth of agar and that air bubbles disappear from the agar before it hardened. All timing was carefully checked.

Plates prepared for assay could be stored at 2° C. for future use; however, there was a loss of accuracy proportional to length of storage time. Such plates were again dried at 50° C. and allowed to attain room temperature before use.

Addition of Assay Material: Each plate selected for assay was superimposed over an area marked on a level table so that assay drops could be placed in the same relative position on each consecutive plate. Following adjustment of the plunger in a 0.25 c.c. tuberculin syringe to the 0.05 c.c. graduation mark, 0.06 c.c. of fluid to be assayed was drawn up into the syringe. The air space between the plunger and assay material established complete visibility of the plunger tip relative to the 0.01 c.c. graduations on the barrel of the syringe and also facilitated complete removal of assay material from the syringe. A 27 gauge, ½ inch needle was placed firmly on the shank of the syringe with the needle's beveled opening down and opposite the graduations on the barrel of the syringe. Precisely 0.01 c.c. of fluid was slowly ejected from the syringe to form a hanging drop on the end of the needle. This drop was touched to the surface of the seeded agar and thus deposited in the appropriate position on the plate. (When the drop was first ejected from the syringe, the instrument had to be held in a horizontal position in order to facilitate adhesion of the drop to the needle.) This procedure was carried out in quintuplet for all samples assayed, one drop on each of five plates. Using this procedure, samples of assay material sufficient to assay in quintuplet could be completed in from ten to fifteen minutes. One individual can successfully complete the operation.

Incubation and Reading of Assay Plates: Assay plates were incubated overnight (approximately seventeen hours) at a temperature ranging from 28 to 30° C., removed from the incubator, and the diameter of the zone of inhibition of growth measured. If desired, results of the assay could be read in eight hours after the material had been added to the plates.* Readings were recorded to the nearest 0.1 millimeter. Values used were within plus or minus 1.0 mm. of one another. For instance, if five readings for any hypothetic value were 21.0, 21.6, 20.9, 21.4, and 22.7, the last value (22.7) was discarded. The average diameter of the zone of inhibition for each level of the standard was plotted on graph paper against units per cubic centimeter for the respective dilutions of the standard to constitute a standard curve (see Figs. 1 and 2). The diameter of the inhibition zone for any unknown assay sample was compared with the standard curve to give results in units of streptomycin per cubic centimeter of fluid.

Any plate-counting apparatus could be used for reading inhibition zones, provided it was equipped with a transparent, graduated film ruled in 1 mm. areas. The diameter could also be read with a millimeter rule. It was found convenient to use a glass plate covered with a positive exposed negative of

*Preliminary results indicated that by incubating the seeded plates for three to six hours at the temperature given, prior to the addition of the assay material, assays could be read in five and two hours, respectively; however, there was a decrease in accuracy directly proportional to time of initial incubation.

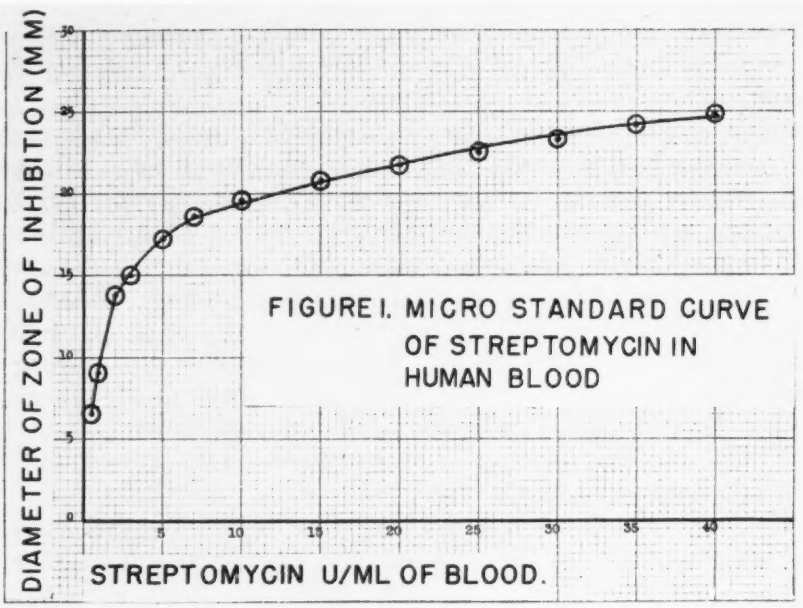
DIAMETER OF ZONE OF INHIBITION (MM)
FIGURE I. MICRO STANDARD CURVE OF STREPTOMYCIN IN HUMAN BLOOD
STREPTOMYCIN U/ML OF BLOOD.

Fig. 1.

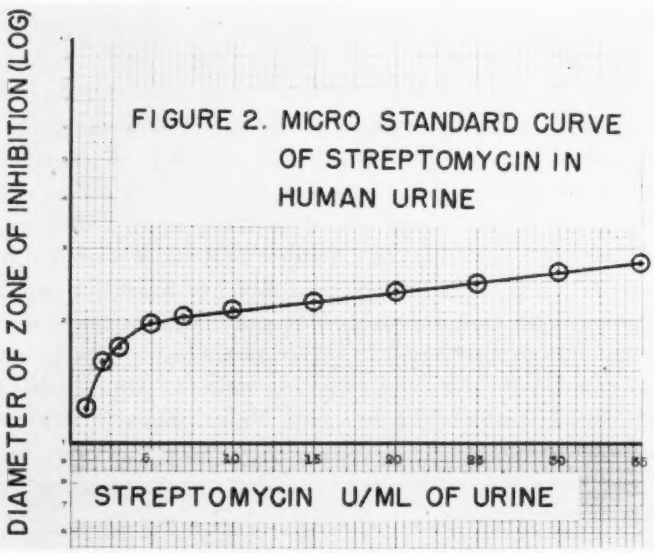
DIAMETER OF ZONE OF INHIBITION (LOG)
FIGURE 2. MICRO STANDARD CURVE OF STREPTOMYCIN IN HUMAN URINE
STREPTOMYCIN U/ML OF URINE

Fig. 2.

1 mm. graph paper on lithographic film.* The diameter of the inhibition zone was superimposed over the plate against a background of light, and the zone thus was read.

*One millimeter graph paper was photographed onto a contrast photographic plate.

Preparation of Standard Curves: Prior to treatment, a 1.5 c.c. sample of body fluid was obtained from the patient for preparing a complete (0 through 40 units) streptomycin standard curve. (Only 1 c.c. need be drawn for a low unitage standard curve, 0.5 through 15 units.) To 0.09 c.c. aliquots of selected fluid, varying concentrations of streptomycin were added and assayed as previously described. (It is imperative that chemically clean 0.1 c.c. serologic pipettes be used for making dilutions and that glassware be thoroughly cleaned each time after use.) The diameter of the resulting zone of inhibition was plotted on graph paper against the corresponding unitage (see Figs. 1 and 2). For example, in following the blood level of streptomycin in a patient, a sample of blood was taken before administration of the drug for constructing a standard curve on blood of that particular person. Blood was collected in lithium oxalate (0.06 c.c. of a 3.3 per cent lithium oxalate solution per 1 c.c. of blood) and mixed. Then 0.09 c.c. of blood was accurately pipetted into each test tube (½ inch in diameter and 1 inch in height). To each of these, 0.01 c.c. of the respective streptomycin solutions of known variable unitage was added. In pipetting blood and standard solutions, the outer surface of the pipette was wiped to remove excess fluid after it was filled and before delivery to the test tube. Blood was allowed to flow slowly from the pipette into the test tube from the 0.01 c.c. mark to its maximum gravitational level; the remainder was gently blown out and the tip of the pipette touched to the side wall of the test tube. Blood without added streptomycin was assayed to determine possible inhibition of *B. subtilis* by the blood itself. Water standards of streptomycin were prepared so that when 0.01 c.c. was added to 0.09 c.c. of blood or other fluid, concentrations of 0.5, 1.0, 2.0, 3.0, 5.0, 7.0, 10.0, and 15.0 units of streptomycin per cubic centimeter resulted for the low unitage curve, and thereafter up through 40 units per cubic centimeter in steps of 5 units for the high unitage curve. (Higher unitages can be approximated.)

Streptomycin standard dilutions of 0.5, 1, 2, 3, 5, 7, 10, and 15 units were placed on one series of five plates and those of 20, 25, 30, 35, and 40 units on another series when using large Petri plates (150 by 20 mm.). When using small Petri plates (100 by 10 mm.) standards from 0 through 10 units were placed on one plate series and standard dilutions from 15 through 30 units on another series of five plates. A third series was used for determining 35 and 40 unit standard dilutions. It is recommended that a complete standard curve be made on each individual prior to initiation of drug therapy.

Manipulation of Body Fluids for Drug-Level Determination.—

Blood: The following technique is recommended to be used in drawing blood for a drug-level determination. Lithium oxalate solution is drawn up into a 0.25 c.c. tuberculin syringe to the 0.15 c.c. mark; the syringe is then inverted (needle end up), and air and remainder of oxalate are extruded, with the exception of that remaining in the shank of the syringe. The residual oxalate solution forms an airtight seal between the plunger and cylinder wall. The plunger is drawn up to the 0.05 c.c. mark. This air space serves as a marker between the blood and plunger, since it leaves the tip of the plunger clearly visible, and also makes it possible to remove all of the blood when assaying.

The finger or other extremity is punctured and 0.06 c.c. of blood *immediately* drawn into the syringe. In drawing blood it is necessary that no air be incorporated into the blood column. This may necessitate squeezing the finger several times until 0.06 c.c. of blood is obtained with no break in the blood column in the syringe. Blood is drawn up into the syringe to the point where the barrel and shank join, being careful that no air is incorporated into the blood now in the syringe. With the shank of the syringe pointing downward, the contents are agitated by gently tapping the side of the cylinder wall with the back of the finger. If the procedure is carefully operated, no air is incorporated into the blood and the oxalate adhering to the syringe wall is adequately mixed with the blood. A 27 gauge, ½ inch needle is tightly placed onto the shank of the syringe with the beveled edge of the needle opposite the graduation marks on the cylinder wall. With the syringe in a horizontal position, the plunger is pushed forward until blood begins to extrude from the needle, and the tip of the plunger is calibrated on a 0.01 c.c. mark. The tip of the needle is then wiped to remove the trace of blood. Then 0.01 c.c. of blood is extruded from the syringe and the tip of the drop touched to the surface of the agar, thus depositing a drop of blood. It has been found that blood thus drawn can be stored in a refrigerator overnight and assayed the following day. This is convenient for determining drug levels over a period of twenty-four hours or longer, in order that blood may be collected at appropriate intervals and assayed at a later time.

TABLE I. VARIATIONS IN INDIVIDUAL BLOOD MICROSTANDARD CURVES

STREPTOMYCIN ADDED (UNITS PER C.C.)	DIAMETER OF ZONE OF INHIBITION (MM.)					
	DOG			HUMAN BEING		
	DOG A	DOG B	DOG C	PATIENT 1	PATIENT 2	PATIENT 3
0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	6.9	5.3	8.7	6.4	5.5	0.0
1.0	9.0	7.7	12.4	8.0	7.1	6.2
2.0	13.1	11.7	15.1	12.1	10.3	8.0
3.0	15.0	13.3	16.8	14.8	13.2	10.4
5.0	17.4	16.1	18.9	17.2	15.4	13.6
7.0	18.8	17.3	19.4	19.5	16.6	14.6
10.0	19.6	18.1	20.9	20.5	18.5	17.0
15.0	20.9	19.9	22.4	21.5	20.0	18.1

Data for blood standard curves from 0.5 through 15 units in three dogs and three human beings are presented in Table I to illustrate the assay method and to demonstrate the necessity for individual standard curves. A typical micromethod blood standard curve in human beings is shown in Fig. 1. In Fig. 3 are illustrated inhibition zones on a typical plate when assaying blood by the micromethod.

Urine: Streptomycin levels in urine were determined using, for the most part, the same technique as for blood. However, when assaying urine of high unitage (above 40 units), the specimen was diluted with normal urine to the recommended range of 0.5 to 40 units per cubic centimeter for greater accuracy. In preparing a standard curve, 0.1 c.c. of a sample of streptomycin of known unitage was added to 0.9 c.c. of urine for each respective standard

to minimize errors in pipetting. In addition, it was not necessary to maintain a 0.05 c.c. air pocket between the plunger and urine in the syringe when assaying clear specimens. Any convenient amount of urine can be drawn up into the syringe as long as the specimen is assayed in quintuplet. The semilogarithm standard curve on the urine of the patient as presented in Fig. 2 was used to estimate better the very high drug levels.

Assay Technique for Microfilm Method.—This method, developed by the co-author (J. L. K.), was designated as the microfilm method because it requires only a film of seeded agar and is very sensitive for detecting streptomycin in quantities as low as 0.1 unit per cubic centimeter of body fluid.

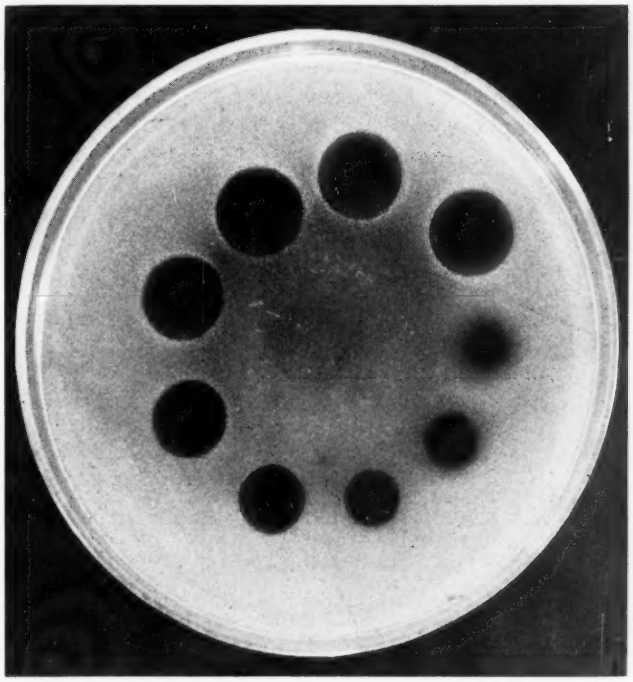

Fig. 3.—Illustrating the type of inhibition zone produced by streptomycin, using the micromethod of assay.

Preparation of Seeded Plates: Difco E-2 Streptothricin Assay Agar was melted, cooled, and maintained at 50° C. in a water bath. It was then seeded with *B. subtilis* spores (approximately 800,000 spores per cubic centimeter of agar), mixed, and maintained in the water bath until the air bubbles emerged from the medium. Approximately 20 c.c. of inoculated agar were added to large sterile Petri plates (150 by 20 mm.). Plates containing the agar were then immediately tipped, and the agar was allowed to pour from the plates into a container for five seconds. The plates were then covered, placed on a flat table, and allowed to harden for ten minutes. This procedure resulted in the formation of a film of agar on the bottom of the plates. The plates were then ready for assaying purposes. Flat-bottomed plates were not necessary when using this technique.

When using small plates (100 by 10 mm.) the same procedure was followed, with the exception that 10 c.c. of seeded agar were added per plate and allowed to flow into a container for the given time (five seconds).

Addition of Assay Material and Incubation and Reading of Assay Plates: The same techniques were used as described for the micromethod.

Preparation of Standard Curves: One cubic centimeter samples of various body fluids have been used for preparing standard curves. The following unitages were used: 0.1, 0.2, 0.3, 0.5, 0.7, 1.0. Although unitages of 2.0, 3.0, and 5.0 could be determined, it was more convenient to assay fluids of such potency by the micromethod. A sample of normal body fluid was assayed to detect possible *B. subtilis* inhibiting factors by the body fluid itself. Techniques and precautions in making standards were the same as for the micromethod; however, the previously designated unitages (0.0 through 1.0 unit) were placed on one plate using either the large (150 by 20 mm.) or small (100 by 10 mm.) Petri plates.

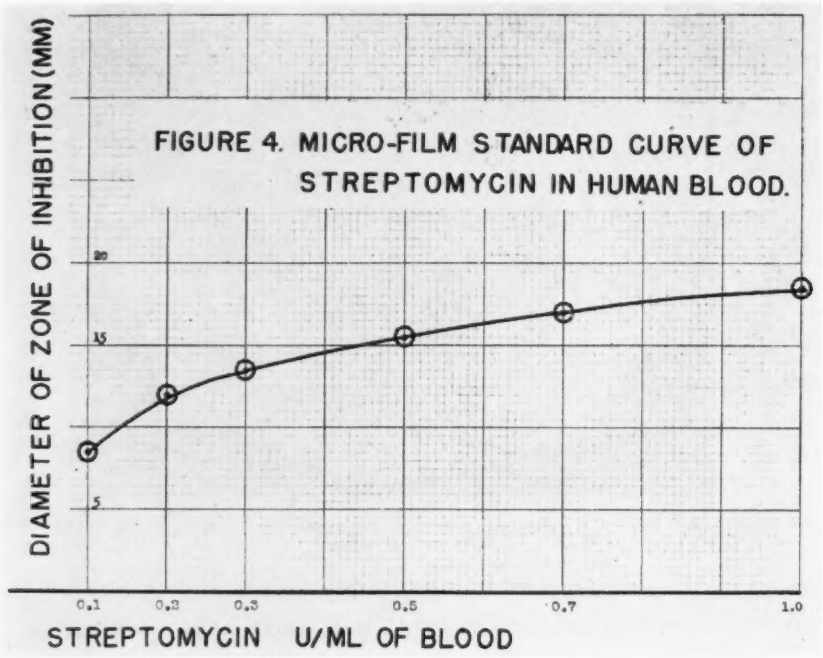

Fig. 4.

In Fig. 4 is illustrated a typical standard curve obtained when using the microfilm method of assaying streptomycin.

Application of Assay Techniques.—Application of these methods for determining streptomycin blood levels in man and in various laboratory animals will be discussed in a succeeding report. The methods are particularly useful where only small amounts of blood can be obtained, such as in mice, guinea pigs, hamsters, and especially in infants where venous puncture is difficult.

In addition, time and material are saved since no pads or cups are required for these methods of assay.

The microfilm assay method was found useful for detecting traces of streptomycin in blood and tissue extracts from animals used for streptomycin excretion studies.

These methods have also been applied successfully to the assay of other antibiotics.

SUMMARY

Two methods, the micro and microfilm techniques for the quantitative determination of streptomycin and other antibiotics in body fluids are described in detail. Using these methods, 1.5 and 1.0 c.c., respectively, of body fluid are necessary for a standard curve and 0.06 c.c. for a level determination in quintuplet. Venous puncture is necessary only for obtaining blood for a standard curve. A drop of blood obtained from a finger or other convenient extremity is used for blood level determinations.

The authors wish to acknowledge their gratitude to Miss Lucy A. Collins, for suggesting development of a micromethod of streptomycin assay, and to Captain Lewis L. Coriell and Dr. Keith H. Lewis, for their suggestions and careful guidance.

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AN IMPROVED PROCEDURE FOR THE DIAGNOSTIC CULTURE OF MAMMALIAN TUBERCLE BACILLI

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WITHIN the past few decades marked strides have been made in the perfection of the diagnostic culture procedure for disclosing the presence of small numbers of mammalian tubercle bacilli. In this short span clinical pathologists and bacteriologists have begun to acknowledge that the culture could efficiently displace the guinea pig or other animal tests[1] for the disclosure of the presence of small numbers of mammalian tubercle bacilli in tuberculous materials from human or animal sources. In spite of the fact that the diagnostic culture procedure is more economical and is superior to the animal test in regard to disclosing the presence of small numbers of moderately or low virulent mammalian tubercle bacilli, even though its superiority is not especially greater for the disclosure of highly virulent human or bovine tubercle bacilli, there are still features of the cultural test which in all probability can be improved materially at present.

The diagnostic culture tests consist of several essential features, the success of the performance of which are significant both in its simplicity and perfection. The first of these features and the part to be particularly elucidated in this study is the preliminary treatment of the specimen to destroy contaminating and undesired microorganisms; while the second equally important feature is the choice of a good nutrient capable of supporting particularly the growth from small plants of mammalian tubercle bacilli.[2, 3]

Historically, the earliest attempts to destroy the contaminating microorganisms made use of such rapidly acting reagents as sodium hypochlorite (Labarraque's solution) with which Griffith experimented in 1914.[4] He found that successful uncontaminated cultures of tubercle bacilli would result if plants were made at short minute intervals and the proper time were hit when the reagent would not destroy the tubercle bacilli, but the contaminants were satisfactorily eliminated from the specimen. Shortly thereafter, Petroff[5] used 3 per cent sodium hydroxide for thirty minutes for the same purpose and with fair success. Later, 3 per cent hydrochloric and 6 per cent sulfuric acid were tried[6] also with success for short period exposure up to an hour or so. In each case, however, the reagent was prepared from liquid materials of uncertain composition or from solids, such as sodium hydroxide, not entirely easy to handle or weigh and subject to change by absorption of carbon dioxide from the air or capable of etching the glass containers in which they were stored. The need for a crystalline solid of high purity and stable composition was attained with the introduction of 5 per cent oxalic acid in 1930.[7] This

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reagent was easily prepared and kept well, ready for use. It was found fairly satisfactory in that it could be used in equal volume of 5 per cent solution to destroy contaminants by exposure up to two hours at incubator temperature, giving a greater time latitude, and in many respects it proved superior to sodium hydroxide. The problem of the nutrient for growing small plants of tubercle bacilli resolved itself around two questions: the choice of a good nutrient and essentially the choice of a simply prepared nutrient.

In Dorset's whole egg medium and its modifications,[8] all the prerequisites for a good nutrient were present, but the possibilities of supporting the growth of small plants remained undisclosed because of lack of adequate test methods in those days. Petroff's gentian violet egg medium contained sufficient retardant dye not only to retard the contaminants but also to retard small plants of tubercle bacilli; and Corper's potato cylinder medium,[9] though a suitable nutrient, was too easily prepared improperly by the technician to become generally popular. Thus, also a number of the more complicated multiple mixture mediums, like Petragnani's, Loewenstein's, Evanoff and Sweany's, Sasano and Medlar's, and other mediums, proved not entirely satisfying because of complications in preparation. Among the simply prepared mediums, the glycerol egg yolk medium[10] when properly prepared appeared to offer simplicity, ease of preparation, and a good nutrient to recommend it; however, it is still open to improvement of certain features such as overcoming the seasonal variation of eggs or the use of improperly stored fresh eggs with the consequent detrimental effect of spoilage products on the growth of the bacilli. All in all, however, the egg yolk medium properly prepared and from fresh eggs proved the simplest and most efficient of nutrient mediums thus far recommended for the growth from small plants of mammalian tubercle bacilli. The matter of coloring the medium for contrast discernment of early colonies was left to the individual choice of the technician, since the retardant effect on contaminants was found to be unimportant and the color was merely added as an inert stain for contrast. As a matter of fact, experienced technicians preferred to use the egg yolk without color and were able to note early colonies just as easily. However, for those desiring a color background, the color became a matter of individual choice covering a wide range from red through green, blue, and even black. The only drawback to dark colors was that a deeply colored egg medium coated the inside of the tube at the observation zone many times and thus defeated the very purpose for which it was added. It was for this reason probably that many technicians chose malachite green or congo red, dyes without intense colors. When analyzed, however, the color matter is not of great significance in the method or efficiency of the nutrient so long as it possesses no retarding action on the growth of small plantings of mammalian tubercle bacilli.[11]

Destroying contaminants or preventing their multiplication during storage of the specimen before adding the reagent for destroying them, however, is of most importance to the ultimate success of the cultural procedure, since it becomes more difficult to clean out contaminations the more heavily the specimen is seeded with them.

With the hope of finding a less active or potent crystalline material obtainable in pure form, which might allow more time for preparation of the specimen and which might even be added to the specimen immediately on collection, a number of chemicals were given consideration for the purpose of replacing the commonly used sodium hydroxide (3 per cent) and oxalic acid (5 per cent) reagents. After consideration of a large number of chemicals in the past, a chemically pure trisodium phosphate used in crude form in laboratories for glassware cleaning purposes was given serious consideration. In preliminary tests it was found that a 10 per cent concentration (equivalent to 23 per cent of the chemically pure hydrated salt $Na_3PO_4 . 12\ H_2O$) added to equal volumes of fine suspensions of mammalian tubercle bacilli proved not only innocuous to the bacilli but was more protective than suspension of the bacilli in 0.9 per cent saline solution for long periods of time. The effects of 3 per cent sodium hydroxide, 5 per cent oxalic acid, and 10 per cent trisodium phosphate on suspensions of tubercle bacilli compared with saline suspensions for intervals up to two weeks of contact are recorded in Table I.

TABLE I. EFFECT OF 3 PER CENT SODIUM HYDROXIDE, 5 PER CENT OXALIC ACID, AND 10 PER CENT TRISODIUM PHOSPHATE ON GROWTH OF TUBERCLE BACILLI

TIME OF EXPOSURE TO REAGENT AT 37° C.	REAGENT											
	SALINE			3% SODIUM HYDROXIDE			5% OXALIC ACID			10% TRISODIUM PHOSPHATE		
	AMOUNT OF TUBERCLE BACILLI IN MILLIGRAMS PER CUBIC CENTIMETER* TREATED											
	1.0	10^{-3}	10^{-6}	1.0	10^{-3}	10^{-6}	1.0	10^{-3}	10^{-6}	1.0	10^{-3}	10^{-6}
0	2†	2	3	2	2	5^{1}‡	2	2	4	2	2	3
2 hr.	2	2	3	2	3	0	2	3	6^{1}	2	2	3
4 hr.	2	2	3	2	3	0	2	3	0	2	2	3
8 hr.	2	2	3	2	3	6^{1}	2	4	0	2	2	3
1 day	2	2	3	2	3	0	3	0	0	2	2	3
2 days	2	2	3	2	3	0	0	0	0	2	2	3
4 days	2	2	5^{2}	2	4	0	0	0	0	2	2	4
1 wk.	2	4^{2}	0	2	4^{2}	0	0	0	0	2	3	4
2 wk.	2	0	0	4	0	0	0	0	0	2	4	6

Note: At one week, 10 per cent trisodium phosphate prevents death of bacilli which normally takes place in saline suspensions.

*To 1.0, 0.001, and 0.000,001 mg. per cubic centimeter of tubercle bacilli in fine suspension was added an equal volume of the reagent. After the incubation period indicated, the NaOH and Na_3PO_4 solutions were neutralized with 5 per cent HCl, and the oxalic acid was neutralized with 3 per cent NaOH before planting. The plantings are necessarily one-half of those indicated.

†The time of contact for killing contaminants in sputum and other specimens with 3 per cent NaOH and 5 per cent oxalic acid is thirty minutes to one hour at 37° Centigrade.

‡The numeral indicates the number of weeks after which growth first appeared; the exponent indicates the number of tubes positive when all three tubes planted did not reveal growth.

It is noted from the data recorded in Table I that the addition of the 10 per cent trisodium phosphate in equal volume to suspensions of tubercle bacilli is less detrimental over a period of two weeks at 37° C. than control saline suspension. Equal volumes of both 3 per cent sodium hydroxide and 5 per cent oxalic acid show detrimental effects which become evident after two hours of contact at 37° C.; this effect is more pronounced for oxalic acid after one day than for sodium hydroxide, although the latter is equally harmful to small numbers of bacilli after as early as two hours' exposure.

To note further the effect of an equal volume of the 10 per cent trisodium phosphate at room temperature (20 to 25° C.) as compared with incubator temperature (37° C.) on tubercle bacilli and the contaminants in positive sputums, six of these sputums were tested following contact with the reagent at intervals up to one week's time. The results of these findings are recorded briefly in Table II.

The data recorded in Table II indicate that the trisodium phosphate in equal volumes of a 10 per cent solution (23 per cent of the hydrated salt $Na_3PO_4.12\,H_2O$) destroys the contaminants in these positive sputums on exposure for one or two days at room temperature and within about one day at incubator temperature (37° C.); whereas, even one week's exposure at incubator temperature (37° C.) was not particularly detrimental to the viability of the tubercle bacilli, although the longer interval of one week's contact retards their growth.

In order to note further the advantages or disadvantages of neutralizing or washing the treated suspensions, the experiment recorded in Table III on positive sputums was performed.

From the results recorded in Table III it is to be noted that either neutralization or washing out the phosphate produces more satisfactory results when compared with the planting in the phosphate as such. When the phosphate is neutralized or washed out, neither the room temperature nor the incubator temperature appear to be especially detrimental, although the incubator temperature is slightly more so. However, it is significant that the bacilli in sputum can be kept in the phosphate mixture for up to one week without preventing subsequent recovery of the tubercle bacilli planted on a good nutrient medium such as the glycerol egg yolk medium.

The superiority of the one-day treatment at 37° C. with an equal volume of 10 per cent trisodium phosphate compared with the standard one-hour treatment at 37° C. with an equal volume of 5 per cent oxalic acid is illustrated from the examination of eighteen microscopically negative smear and guinea pig test negative specimens of sputum from custodial cases. These disclosed three positive findings for one highly virulent and two moderately virulent strains of human tubercle bacilli as presented with the data recorded in Table IV.

Human feces are difficult to prepare satisfactorily for cultural purposes, and Table V presents a comparison of oxalic acid (5 per cent) and sodium hydroxide (3 per cent), with different time exposures from one day to one week at 37° C., to the trisodium phosphate (10 per cent).

It is noted from the data recorded in Table V that while both oxalic acid and trisodium phosphate are more efficient in clearing up the contaminants in feces than is the sodium hydroxide, the trisodium phosphate proved superior to both the oxalic acid and sodium hydroxide in that a one-week exposure at 37° C. succeeded in a number of cases in removing the contaminants from the human feces. However, no satisfactory method has been found for recovering tubercle bacilli from feces, and this phase will be reported on fully in a subsequent detailed paper.

TABLE II. EFFECT OF 10 PER CENT TRISODIUM PHOSPHATE* ON CONTAMINANTS AND ISOLATION OF MAMMALIAN TUBERCLE BACILLI FROM POSITIVE SPUTUMS

TIME OF CONTACT WITH REAGENT†	ROOM TEMPERATURE												INCUBATOR TEMPERATURE (37° C.)											
	NUMBER DESIGNATION OF SPUTUM																							
	1		2		3		4		5		6		1		2		3		4		5		6	
	+*	C†	+	C	+	C	+	C	+	C	+	C	+	C	+	C	+	C	+	C	+	C	+	C
1 day	3‡	③§	3	0	3	0	3	0	3	1	3	③	3	2	3	0	3	0	3	0	3	1	3	0
2 days	3	1	3	0	3	1	3	1	3	1	3	1	4	0	3	0	3	1	3	0	3	0	3	1
4 days	3	0	3	0	3	0	3	0	3	0	3	1	4	1	4	0	4	0	3	0	4	0	3	0
1 wk.	3	0	3	0	3	0	3	0	3	0	3	2	7	0	4	0	5	0	3	0	5	0	5	0

Note: Ten per cent trisodium phosphate (Na_3PO_4) is equivalent to 23 per cent trisodium phosphate weighed with the water of crystallization ($Na_3PO_4 . 12\ H_2O$).

*+, Cultures positive for tubercle bacilli.

†C, Contaminations of three tubes planted.

‡The number of weeks after which growth of tubercle bacilli first appeared.

§A ○ around a number means contaminated after the culture had been positive for tubercle bacilli.

TABLE III. POSITIVE CULTURES AND CONTAMINATIONS RESULTING FROM TRISODIUM PHOSPHATE TREATMENT OF POSITIVE SPUTUMS FOLLOWING NEUTRALIZATION OR WASHING OF TREATMENT MIXTURE BEFORE PLANTING

TIME OF EXPOSURE TO REAGENT	ROOM TEMPERATURE															INCUBATOR TEMPERATURE (37° C.)													
	SPUTUM NUMBER																												
	1			2			3			4			5			1			2			3			4			5		
	A*	B†	C‡	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1 day	3^{1}§	3^{1}	3^{2}	3^{2}	3^{1}	2^{0}	3^{0}	3^{0}	3^{2}	0^{3}	0^{3}	6^{2}	3^{0}	3^{1}	3^{0}	3^{0}	3^{1}	3^{0}	3^{0}	3^{0}	3^{0}	3^{0}	3^{1}	3^{1}	3^{2}	0^{3}	4^{1}	3^{0}	3^{0}	3^{0}
2 days	3^{0}	3^{2}	3^{0}	3^{0}	3^{0}	3^{0}	3^{1}	3^{1}	3^{1}	4^{1}	4^{2}	0^{3}	3^{0}	3^{0}	3^{1}	4^{0}	3^{0}	3^{1}	4^{1}	3^{1}	3^{1}	3^{2}	3^{0}	3^{0}	4^{1}	3^{0}	4^{2}	3^{0}	3^{0}	3^{0}
4 days	3^{0}	3^{0}	4^{2}	3^{0}	3^{0}	3^{0}	3^{0}	3^{1}	3^{1}	0^{3}	0^{3}	0^{3}	4^{1}	3^{1}	3^{0}	4^{0}	4^{0}	3^{1}	5^{0}	3^{0}	3^{1}	4^{0}	3^{0}	3^{0}	0^{1}	5^{1}	4^{0}	4^{0}	3^{0}	3^{0}
1 wk.	3^{0}	3^{0}	0^{3}	3^{0}	3^{0}	3^{2}	3^{0}	3^{1}	0^{3}	4^{2}	3^{1}	0^{3}	3^{0}	3^{0}	3^{1}	5^{0}	3^{0}	3^{1}	4^{0}	3^{1}	3^{0}	4^{0}	3^{0}	3^{0}	0^{1}	4^{0}	0^{2}	4^{0}	3^{0}	3^{0}

*A, Not neutralized.

†B, Neutralized.

‡C, Washed, not neutralized.

§Number, Weeks in which positive culture first appeared; exponent, number of tubes of three which were contaminated.

TABLE IV. ISOLATION OF MAMMALIAN TUBERCLE BACILLI FROM MICROSCOPIC SMEAR AND GUINEA PIG NEGATIVE SPUTUMS

PATIENT	RESULTS OF CULTURE TEST: 5% OXALIC ACID TREATMENT (1 HOUR AT 37° C.)	RESULTS OF CULTURE TEST: 10% TRISODIUM PHOSPHATE TREATMENT (1 DAY AT 37° C.)	VIRULENCE TEST WITH BACILLI ISOLATED[12]
W. E.	0	0	
S. H.	0	0	
A. B.	0	0	
G. U.	0	+	Moderately virulent
G. R.	0	0	
H. A.	0	0	
C. O.	0	+	Highly virulent
R. I.	0	0	
T. O.	0	0	
E. K.	0	0	
K. E.	0	+	Moderately virulent
B. O.	0	0	
R. A.	0	0	
S. E.	0	0	
B. A.	0	0	
R. O.	0	0	
M. I.	0	0	
O. Y.	0	0	

Note: We are indebted to Dr. Fritz Rosenberg, Medical Director of the Ex-Patients' Tubercular Home of Denver, for these sputums which were obtained from old custodial cases.

TABLE V. EFFECT OF DIFFERENT PREPARATION REAGENTS ON CONTAMINANT MICROORGANISMS IN HUMAN FECES OBTAINED FROM PATIENTS WITH POSITIVE SPUTUMS

FECES NUMBER	REAGENTS USED TO DESTROY MICROORGANISMS: 2 VOL. 5% OXALIC ACID TO 1 VOL. FECES FOR 1½ HR. AT 37° C.	2 VOL. 3% SODIUM HYDROXIDE TO 1 VOL. FECES FOR 1½ HR. AT 37° C.	2.5 VOL. 10% TRISODIUM PHOSPHATE TO 1 VOL. FECES FOR 1 DAY AT 37° C.	2.5 VOL. 10% TRISODIUM PHOSPHATE TO 1 VOL. FECES FOR 3 DAYS AT 37° C.	2.5 VOL. 10% TRISODIUM PHOSPHATE TO 1 VOL. FECES FOR 1 WK. AT 37° C.
9646	4*	4	3	2	0
9391	1	4	4	4	2
9590	2	4	4	4	0
9537	1	4	4	4	1
9507	4	4	4	4	2
9529	3	4	4	4	1

*The number of contaminated tubes of 4 planted with neutralized treated feces.

TABLE VI. EFFECT OF SODIUM HYDROXIDE, OXALIC ACID, AND TRISODIUM PHOSPHATE TREATMENT UPON MOLDS AND ACID-FAST SAPROPHYTE

MICROORGANISM	REAGENT	TIME OF CONTACT AT 37° C. (HR.)	TEMPERATURE OF CULTURE TEST FOR VIABILITY: ROOM	37° C.
	3% sodium hydroxide	1	+*	0†
		2	0	0
Penicillium notatum	5% Oxalic acid	4	+	+
	10% Trisodium phosphate	1	+	0
		24	0	0
Actinomyces griseus	3% NaOH	2	+	+
	5% Oxalic acid	1	0	0
	10% Trisodium phosphate	24	+	+
Acid-fast saprophyte (Day)	10% Trisodium phosphate	1½	0	0

*Growth of the test mold or acid-fast saprophyte.
†Absence of growth.

In order to note the effect of sodium hydroxide, oxalic acid, and trisodium phosphate on *Penicillium notatum* and *Actinomyces griseus,* as well as on an acid-fast saprophyte, the experiment recorded in Table VI was performed.

The findings recorded in Table VI indicate that the trisodium phosphate (10 per cent) is capable of destroying *P. notatum* within one hour at 37° C. and twenty-four hours at room temperature, and sodium hydroxide (3 per cent) does this within one hour at 37° C. and two hours at room temperature; however, oxalic acid (5 per cent) applied for four hours at 37° C. is unable to destroy this organism. With *A. griseus* just the reverse is true in that oxalic acid (5 per cent) is capable of destroying this organism in one hour at room temperature. Sodium hydroxide (3 per cent) for two hours at 37° C. and trisodium phosphate (10 per cent) for one day at 37° C. are inactive. Acid-fast saprophyte (Day) is destroyed by one and one-half hours' contact with 10 per cent trisodium phosphate. These findings suggest that it may be necessary at times to use both alkali (trisodium phosphate) and acid (oxalic acid) treatment to destroy certain mold contaminants, if we can draw conclusions from the tests with *P. notatum* and *A. griseus.*

SUMMARY AND CONCLUSIONS

1. A new reagent, trisodium phosphate, has been tested to determine its value in comparison with the standard reagents, including oxalic acid and sodium hydroxide, for destroying contaminants in tuberculous materials in preparation for the cultural diagnoses of tuberculosis and for isolating mammalian tubercle bacilli.

2. Trisodium phosphate is a reagent which is a pure crystalline chemical material of standard composition. In 10 per cent solution (23 per cent of the $Na_3PO_4.12\,H_2O$), it can remain in contact with tubercle bacilli for up to a week at room temperature without destroying small numbers of tubercle bacilli, but it destroys the undesirable contaminants in sputum within twenty-four hours at 37° C. or within several days to a week at room temperature.

3. Trisodium phosphate as a reagent for destroying contaminations found in tuberculous materials can also be placed in the receptacles used for collecting the tuberculous specimen and thus immediately prevents the development of molds and undesired contaminants. This procedure assures better materials for culturing mammalian tubercle bacilli and therefore the loss of valuable specimens.

4. The time required for destroying contaminants by the trisodium phosphate is one day at 37° Centigrade. This also adds to the convenience with which the technician can prepare his specimens for culture, since there is no necessity for haste in the preparations such as is required with the previously used reagents sodium hydro- le and oxalic acid.

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MODIFICATION OF THE FAUST METHOD IN THE DETECTION OF CYSTS AND OVA

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THE Faust method for the concentration and flotation of cysts and ova has proved valuable as a diagnostic aid in certain infections of the gastrointestinal tract, particularly amebiasis.[1] During the summer and fall of 1945, ever-increasing troops with amebiasis were arriving from the Pacific Theatre of Operations. The diagnosis on some of these cases was confirmed immediately by the direct smear method alone due to the great number of *Endamoeba histolytica* cysts present. Nevertheless, some clinically diagnosed cases continued to exist in which negative results were consistently obtained both by the direct smear and the Faust zinc sulfate flotation methods. Experience had taught us that the latter is the better method in the search for cysts, so we began to suspect that our negative results occurred because cysts were inconsistently present in the stools or because the clinical diagnosis was incorrect. As recommended by Faust and others,[1] patients were purged with a saline cathartic and subsequent stools were examined.[2] Both vegetative and cyst forms were recovered in some of the cases, the latter forms being found mostly by the zinc sulfate method. In spite of the purgation, only a few cysts were found. The remaining negatives were examined daily for a week and remained negative even after purgation. Thinking that in the routine method of Faust the stool sample might be too small, we decided to modify the method by combining it with my method for detecting schistosoma which had given excellent results in over 25,000 examinations of 800 patients with schistosomiasis japonica.[3] The modified method is as follows.

THE MODIFICATION

1. Place about 10 Gm. of feces in a 125 c.c. Erlenmeyer flask containing about 75 c.c. of warm water (about 40° C.); stopper the flask and shake until emulsified.

2. Strain through 1 layer of wet gauze into a 50 c.c. centrifuge tube (teated bottom).

3. Centrifuge for one minute at 2,500 r.p.m. and pour off supernatant fluid. Add warm water to the sediment from a regulated faucet (force of the water will disperse the fecal particles and cleanse them) and centrifuge again at the same time and speed. Decant supernatant fluid; repeat one more washing and centrifugation; again decant supernatant fluid.

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4. Add about 10 c.c. of warm water and agitate sediment by shaking; then pour quickly all of contents into a 15 c.c. centrifuge tube. Centrifuge for one minute at 2,500 r.p.m. and decant supernatant fluid.

5. Add about 10 c.c. of zinc sulfate solution (33 per cent, specific gravity 1.180) to the sediment and stir with applicator sticks. Add zinc sulfate nearly to top of tube. Centrifuge for one minute at 2,500 r.p.m. Ova and cysts will float to the surface.

6. Take several loopfuls (bacteriologic loop) from surface and place on slide; add 1 drop of D'Antoni's or Lugol's iodine and cover with a cover slip. Examine for ova of helminths and cysts of protozoa under low-power objective of microscope.

DISCUSSION OF METHOD

This method varies from the original method (Faust's) as follows:

1. A much larger sample of feces is utilized, thereby increasing possibility of detection in lightly infested cases or when cysts and ova are rare in the stool. Cysts and ova quite often are not evenly distributed and a large sample is desirable. Cysts, particularly, may exist in "packets" and can be missed from a remote small section. If only one cyst or ovum is present on a slide (this quite often occurs), the average technician may experience great difficulty in finding it.

2. The use of warm water (about 40° C.) certainly has advantages over the use of regular or so-called cold water. If cold water is used, washing of the sediment can be accomplished and most of the bile coloring matter is removed; yet, this type of washing is not complete because a loose scum remains with the sediment and has a tendency to float when the zinc sulfate is added, thereby impairing the clear field which should exist for the microscopic examination. The so-called fatty type of stool offers practically the same difficulty, and the use of cold water is not of too much value. On the other hand, the use of warm water eliminates a high percentage of the scum or fats. After the water is added the scum rises to the surface within one minute, remains floating after the centrifugation, and eventually is discarded with the supernatant fluid. Each additional washing brings up less scum until it is practically all discarded. When the zinc sulfate now is added, the surface of the liquid is fairly clean and makes a good field for microscopic examination. Some workers have suggested the use of cold physiologic saline for the washing, but this is also inferior to plain warm water.

3. The initial use of the large 50 c.c. tube instead of the smaller test tube is first of all important because it is necessary to receive a large sample from the original 10 Gm. of stool. Our purpose would be defeated if 10 Gm. of stool were originally emulsified and only 5 to 10 c.c. of the emulsified fluid examined. With the teated bottom of the 50 c.c. tube there is less tendency for cysts or ova to be lost in the decanting process. With the larger tube the proportion of sediment to the size of the tube decreases the number of washings and centrifugations in arriving at a clear supernatant fluid; three or four washings usually give this desired result and a clean zinc sulfate preparation.

CONCLUSIONS

A modification of the Faust method is described, which increases the yield of cysts and ova as well as *Strongyloides larvae*. It is of particular value when cysts, ova, and larvae are rare in the fecal specimens.

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A RAPID PYRIDINE SILVER STAIN FOR NERVOUS TISSUE AND RETICULAR FIBERS

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THE explosive nature of ammoniacal silver solutions[1-3] and the technical difficulties attendant upon their use for staining nervous tissue elements and reticular fibers have led to the development of a simple and rapid silver stain for these purposes. Pyridine is employed instead of ammonium hydroxide in the preparation of the silver stain, rendering the solution easy to prepare and obviating the danger of explosions. For routine purposes the method is useful for demonstrating axons and dendrites of neurons, Alzheimer's neurofibrillary change, fibrillary astrocytes, and reticular fibers.

I. To Demonstrate Axons, Dendrites, and Alzheimer's Neurofibrillary Change.—

1. Collect formalin-fixed frozen sections about 25 μ each in distilled water containing a trace of ammonium hydroxide.

2. Transfer sections to silver pyridine solution for about six to ten minutes. Solution is prepared by mixing the following together until clear:

Silver nitrate, 1 per cent	2 c.c.
Potassium carbonate (anhydrous), 1 per cent	1.5 c.c.
Pyridine (pure)	0.16 c.c.

3. Transfer sections directly to excess 10 per cent formalin neutralized with calcium carbonate for about one minute.

4. Float sections onto slides, dehydrate through alcohols and xylols in usual manner, and mount in balsam. Axons, dendrites, Alzheimer's pathologic neurofibrils, and nuclei stain black against yellow background.

II. To Demonstrate Fibrillary Astrocytes.—The method is exactly the same as for nerve cell fibers, except that fixation is carried out in formalin bromide (2 Gm. of ammonium bromide in 100 c.c. of 10 per cent formalin). Processes of fibrillary astrocytes stain black, while axons and dendrites are usually poorly stained.

III. To Demonstrate Reticular Fibers.—

1. Hydrate routine formalin-fixed paraffin sections in usual manner.

2. Transfer slides to potassium permanganate ¼ per cent solution for five minutes. Rinse in tap or distilled water.

3. Transfer to oxalic acid 5 per cent solution for five minutes. Rinse well in distilled water.

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4. Transfer to silver pyridine solution, prepared by mixing the following together successively until clear, and stain for seven minutes:

Silver nitrate, 1 per cent	20 c.c.
Potassium carbonate (anhydrous), 1 per cent	15 c.c.
Pyridine (pure)	1.6 c.c.

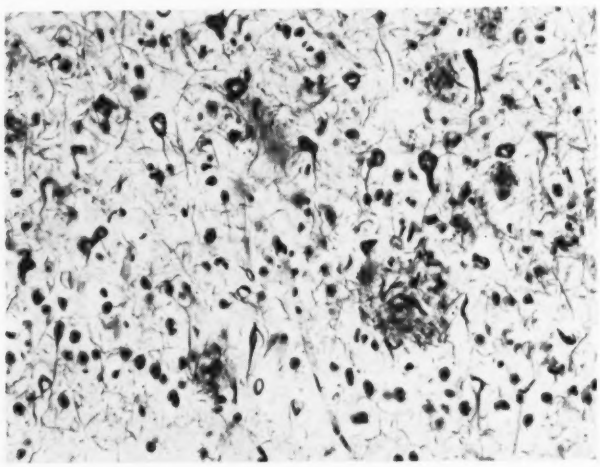

Fig. 1.—Alzheimer's neurofibrillary change in cerebral cortex. Pyridine silver stain (section 25 μ), seven minutes $\times$200.

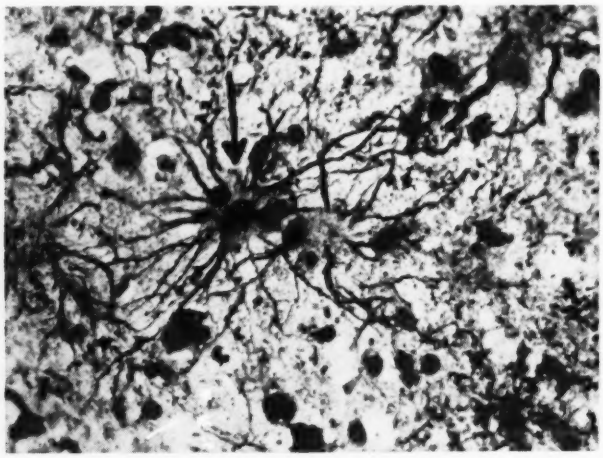

Fig. 2.—Fibrillary astrocytes. Focal level on central astrocyte (section 25μ) $\times$400. Pyridine silver stain, nine minutes.

5. Transfer directly to excess of 10 per cent formalin (neutralized with calcium carbonate) for about one minute.

6. Dehydrate in alcohols and xylols in usual manner and mount in balsam.

Reticular fibers stain black. Nuclei are not stained black by silver.

The silver pyridine solution is prepared fresh just before staining and may be used for one to three hours. Merck's and Eastman Kodak's chemically pure

pyridine have been found satisfactory. Toning with gold chloride and the application of counterstains may be employed in the manner described in texts on staining methods. For routine purposes this is not usually necessary.

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THE QUANTITATIVE KAHN TEST: RESULTS WITH 2.5 PER CENT AND 0.9 PER CENT SALT SOLUTION COMPARED WITH THE DEGREE OF REACTIONS OF THE STANDARD KAHN TEST

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SINCE 1940 the routine laboratory tests for syphilis in the Division of Laboratories of the Tennessee Department of Public Health have been the presumptive and standard Kahn tests for blood specimens and the Kolmer complement fixation test for spinal fluid. All tests are performed in exact accord with outlines of methods by author serologists.[1, 2] Antigen for the Kolmer complement fixation test is purchased from commercial sources. Antigen for the Kahn standard test is purchased from the University Hospital, Ann Arbor, Michigan. Both tests have been included in annual national evaluation studies.

With the advent of rapid treatment centers in the state, the need became acute for quantitative tests to determine the serologic status of patients with syphilis before and after treatment. At first these quantitative tests were sent to Dr. Reuben L. Kahn at Ann Arbor. Later the Central Laboratory of the Division of Laboratories was able to start making quantitative Kahn tests following the method given by Kahn[3] in Supplement 11, Venereal Disease Information. For a while duplicate specimens were sent by the treatment centers to Kahn's Laboratory and to the Central Laboratory. Titers on these duplicate specimens were quite different, with Kahn's Laboratory obtaining consistently higher titers than the Central Laboratory. Investigation revealed that Kahn's Laboratory was using 2.5 per cent salt solution in dilutions and in the performance of the test, while the Central Laboratory was using 0.9 per cent salt solution. The Central Laboratory changed to 2.5 per cent salt solution, using a 6:1 serum antigen ratio, and no further significant differences were noted in the titers obtained in the two laboratories. The Central Laboratory took over the examination of rapid treatment center specimens for quantitative tests in 1944, as a special procedure.

The numbers of specimens handled as special procedures for the treatment centers have increased steadily, and at present the Central Laboratory is making over 4,000 such quantitative tests each month. This increasing load, in addition to increases in routine serologic work in a serologic section of limited space and personnel, has been of considerable concern. To cut down to some extent on the work involved, thought was given to the elimination of the standard Kahn test as a preliminary to the quantitative Kahn test. The standard test seemed a very poor screen for the quantitative test on specimens from previously diagnosed patients with syphilis when the work involved in the standard test was considered in relation to the numbers of such specimens eliminated from the quantitative test. As all specimens for the quantitative Kahn test were from

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previously diagnosed untreated patients, or from previously diagnosed treated patients, it was decided that the standard Kahn test could be safely eliminated as a preliminary to the quantitative Kahn test if the first tube of the quantitative test contained undiluted serum with the usual dilutions of serum in the remaining tubes. When this was tried in practice, it was found that certain sera gave negative or doubtful reactions in the undiluted state but gave positive reactions in one or more of the dilutions. The standard Kahn test applied to these sera gave negative or doubtful reactions.

We have considered the standard Kahn test as quantitative in its differentiation of negative and weakly positive sera with the quantitative Kahn test in direct correlation and of value in determining the potency of strongly positive sera undifferentiated by the standard test. This conception is apparently in error when 2.5 per cent salt solution is used in the quantitative test, as it is not reasonable to consider the number of specimens we encountered that gave titers as due to prozones.

The fact that titers were obtained on negative or doubtful standard Kahn tests was disturbing, as patients are returned to rapid treatment centers one year after initial treatment if they show 10 or more Kahn reacting units (2 plus reaction in 1:2.5 dilution or higher). Before retreatment at medical centers, the serologic record of the individual is reviewed: if the serologic titer has leveled off at 10 units or more retreatment is given; if the record shows a consistent, continued falling titer the patient is re-evaluated at monthly intervals for two months; if further drops occur the patient is not retreated and is followed for additional periods.

The plan of eliminating the standard Kahn test as a preliminary to the quantitative Kahn test was abandoned as was the use of 2.5 per cent salt solution in the quantitative Kahn test. The quantitative Kahn test employing 2.5 per cent salt solution (6:1 serum antigen ratio) was applied experimentally to specimens received for routine diagnostic test. The specimens titrated were unselected other than that those chosen were specimens with sufficient serum giving normal types of reaction in the following groups: (1) negative standard Kahn; (2) doubtful standard Kahn; (3) 2 plus standard Kahn; (4) 3 plus standard Kahn; and (5) 4 plus standard Kahn. No specimens giving atypical or reverse reactions were included, as such abnormal reactions are usually given by highly potent sera which are likely to yield prozone reactions. Results obtained with the quantitative Kahn test, employing 2.5 per cent salt solution on these five groups of specimens, are given in Table I.

For comparative purposes the quantitative Kahn test employing 0.9 per cent salt solution (6:1 serum antigen ratio) was made on approximately the same numbers of routine specimens giving normal types of 3 plus and 4 plus reactions. No specimens giving atypical or reverse reactions were included. Results obtained with the quantitative Kahn test employing 0.9 per cent saline on these two groups of specimens are given in Table II.

DISCUSSION

It is understood that the quantitative Kahn test is intended only for use with sera giving strongly positive reactions with the standard Kahn test. How-

TABLE I

STANDARD KAHN TEST RESULTS	TOTAL SPECIMENS	QUANTITATIVE KAHN TEST—2.5 % SALT SOLUTION (6:1 SERUM ANTIGEN RATIO)									
		NEGATIVE IN ALL DILUTIONS 0, 1, 2, 3, OR 4 KAHN UNITS BASED ON STANDARD TEST	POSITIVE 1:2.5 DILUTION 10 KAHN UNITS	POSITIVE 1:5 DILUTION 20 KAHN UNITS	POSITIVE 1:10 DILUTION 40 KAHN UNITS	POSITIVE 1:20 DILUTION 80 KAHN UNITS	POSITIVE 1:40 DILUTION 160 KAHN UNITS	POSITIVE 1:80 DILUTION 320 KAHN UNITS	POSITIVE 1:100 DILUTION 400 KAHN UNITS	POSITIVE 1:150 DILUTION 600 KAHN UNITS	POSITIVE 1:200+ DILUTION 800+ KAHN UNITS
Negative (−)	745	604	55	30	27	10	18	1	0	0	0
Doubtful (± and 1+)	690	165	212	196	85	25	4	0	0	1	2
Positive (2+)	551	57	120	186	137	39	11	0	0	0	1
Positive (3+)	527	33	80	195	165	46	7	0	0	0	1
Positive (4+)	1035	4	10	68	280	320	308	14	11	6	14

TABLE II

STANDARD KAHN TEST RESULTS	TOTAL SPECIMENS	QUANTITATIVE KAHN TEST—0.9 % SALT SOLUTION (6:1 SERUM ANTIGEN RATIO)									
		NEGATIVE IN ALL DILUTIONS 3 OR 4 KAHN UNITS BASED ON STANDARD TEST	POSITIVE 1:2.5 DILUTION 10 KAHN UNITS	POSITIVE 1:5 DILUTION 20 KAHN UNITS	POSITIVE 1:10 DILUTION 40 KAHN UNITS	POSITIVE 1:20 DILUTION 80 KAHN UNITS	POSITIVE 1:40 DILUTION 160 KAHN UNITS	POSITIVE 1:80 DILUTION 320 KAHN UNITS	POSITIVE 1:100 DILUTION 400 KAHN UNITS	POSITIVE 1:150 DILUTION 600 KAHN UNITS	POSITIVE 1:200+ DILUTION 800+ KAHN UNITS
Positive (3+)	553	545	8	0	0	0	0	0	0	0	0
Positive (4+)	1041	295	284	227	150	62	19	2	1	1	0

ever, it is felt that this restriction has been imposed because it was considered that sera giving negative or weakly positive reactions with the standard Kahn test would invariably give negative results with the quantitative Kahn test and not because it was thought that confusion would result from the titration of such specimens.

When the quantitative Kahn test employing 2.5 per cent saline (Table I) was applied to sera giving negative standard Kahn reactions, 19 per cent of these specimens gave a titer of 10 or more Kahn reacting units. Titers of 10 or more Kahn reacting units were obtained with 76 per cent of specimens giving doubtful standard Kahn reactions. Titers of 10 or more Kahn units were obtained with 89 per cent of 2 plus specimens, 93 per cent of 3 plus specimens, and over 99 per cent of 4 plus specimens.

When the quantitative Kahn test employing 0.9 per cent saline (Table II) was applied to approximately the same numbers of 3 plus and 4 plus specimens, it was found that less than 2 per cent of 3 plus specimens and only 71 per cent of 4 plus specimens gave titers of 10 or more Kahn units.

It seems logical to expect that a certain number of specimens giving a 4 plus reaction are only potent enough to produce this 4 plus reaction. The value of the quantitative test is in the differentiating or grading of these 4 plus or strongly positive specimens into those that are 4 plus, 40 plus, or 400 plus.

CONCLUSIONS

The results of this study indicate that the quantitative Kahn test employing 2.5 per cent salt solution is on a different sensitivity level than the standard Kahn test. There appears to be little correlation between the degree of reaction obtained with the standard Kahn test and the titer obtained with the quantitative Kahn test employing 2.5 per cent salt solution. The standard Kahn test and the quantitative Kahn test employing 0.9 per cent salt solution (6:1 serum antigen ratio) seem to be on the same sensitivity level. There appears to be a direct relation between the reaction obtained with the standard Kahn test and the titer obtained with the quantitative Kahn test employing 0.9 per cent salt solution. From our observations it seems that the exaggeration of the potency of syphilitic serum through the use of an overly sensitive quantitative test is of doubtful value and can be justified with difficulty where patients are retreated on the basis of an arbitrary low titer after one year.

SUMMARY

Titers obtained in quantitative Kahn tests employing 2.5 per cent and 0.9 per cent salt solution are each compared with results obtained in the standard Kahn test on sera giving normal typical reactions. Tables are presented showing differences.

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A NOTE ON QUANTITATIVE KAHN TESTS EMPLOYING 0.9 AND 2.5 PER CENT SALT SOLUTION SYSTEMS

REUBEN L. KAHN, SC.D.
ANN ARBOR, MICH.

INQUIRIES are occasionally received in this laboratory regarding the technique of the quantitative Kahn test.* This test is not performed identically by different workers. Some employ serial dilutions of serum with 0.9 per cent NaCl solution and others employ serial dilutions of serum with 2.5 per cent NaCl solution. The quantitative test with 2.5 per cent salt solution is generally more sensitive than that with 0.9 per cent salt solution to the extent of approximately one or two additional serum dilutions. Thus, a serum that gives an end point in a 1:20 dilution with 0.9 per cent salt solution may give an end point in 1:30 or 1:40 dilution with 2.5 per cent salt solution.

In the reading of the results of the quantitative test with 2.5 per cent salt solution, emphasis has been given to the fact that the negative reactions tend to show clouding and precipitation on standing. This occurs even when they stand for relatively short periods, such as five to ten minutes. For this reason the results should be read without delay after the addition of the 0.5 c.c. amounts of 2.5 per cent salt solution, following the three-minute shaking period. Apparently it is this concentration of salt solution which causes the clouding and precipitation on standing. Shaking by hand for a few seconds disperses such precipitates and renders the tests clear again. Those workers who have not paid strict attention to this shaking of the tubes just before reading soon found themselves reading particles due to standing of the tests in the presence of the 2.5 per cent salt solution.

Some years ago we recommended the quantitative technique with the 2.5 per cent salt solution, because studies in this laboratory indicated that the salt concentration favors syphilitic reactions and renders them more sensitive than 0.9 per cent salt solution. The increased sensitivity seemed desirable because the quantitative test was then used largely in known cases of syphilis in the follow-up of therapy.

There are, however, points in favor of the use of quantitative Kahn tests with 0.9 per cent salt solution. During recent years quantitative tests have been used as an aid in diagnosis; hence, the high sensitivity of the 2.5 per cent system may also introduce an element of nonspecificity in certain cases. The tests with the 0.9 per cent system do not show a tendency toward clouding on standing and, therefore, do not require agitation immediately before reading. Furthermore, quantitative results with the 0.9 per cent system show closer correlation with the results of the standard Kahn test than quantitative results

From the Serology Laboratory, University Hospital, University of Michigan.
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*Kahn, R. L.: Technique of Standard Kahn Test and of Special Kahn Procedures, Ann Arbor, 1946, University of Michigan Press, p. 22.

with the 2.5 per cent system. Thus, in order to avoid confusion between the 1 to 4 plus terminology of the standard Kahn test and the unit terminology of the quantitative test, we arbitrarily correlated the two terminologies as follows: One plus, 2 plus, and 3 plus in the standard test were arbitrarily considered as 1 unit, 2 units, and 3 units in the quantitative test. Sera giving 1, 2, or 3 plus reactions with the standard Kahn test generally give negative reactions on serial dilution of serum. Occasionally it will be found that a serum giving a 2 plus reaction, and particularly a 3 plus reaction in the standard test, will give a titer of 10 or 20 or higher in the quantitative test. The results are then reported to physicians with the explanation of this exceptional behavior of the serum. However, when performing quantitative tests with the 2.5 per cent NaCl system with weakly positive sera, greater numbers will be found to give quantitative titers. Hence, 1, 2, or 3 plus reactions with the standard test cannot, strictly speaking, be considered as equivalent to 1, 2, or 3 units in the quantitative test.

In this laboratory quantitative tests with both the 0.9 per cent and 2.5 per cent NaCl systems are employed in most cases. This scheme is obviously not feasible for general application. As to the choice between the two quantitative techniques, we believe that in view of the fact that quantitative Kahns are no longer used almost exclusively as serologic checks on therapy in syphilis, but are used increasingly as an aid in the establishment of a diagnosis, the 0.9 per cent NaCl system should be preferred.

A SIMPLE AND SAFE METHOD FOR THE GASTRIC INSTILLATION OF FLUIDS IN THE RAT

HARRY SHAY, M.D., AND MARGOT GRUENSTEIN
PHILADELPHIA, PA.

THE rat has been used as an experimental animal in the field of nutrition for many years. More recently it has gained an important place for the study of problems in digestion. Both of these fields of application have made it most desirable that a rapid and safe method for introducing measured amounts of fluid into the stomach be devised.

As early as 1908 Marks[1] employed a silk-rubber catheter for the stomach tube feeding of mice. His method required two operators. In 1943 Ferrill[2] described the use of a metal cannula made from an injection needle carrying a small perforated brass knob. He claims safety and speed for the method. Methods utilizing a rubber catheter always involve the problem of preventing the rat from biting the tube. Most operators[3, 4] employed a wooden mouth gag with a central hole for the passage of the catheter. We have tried this method and found it cumbersome and time consuming. The wooden mouth gag often hurts the animal, causes it to struggle, and not infrequently injures the mouth of the animal. We found the suspension method described by Machella and Griffith[4] too time consuming to be used for large groups of animals. We have had no experience with the procedure recently reported by Lehr,[5] but from its description it involves many more steps than the very simple and safe method we have evolved in our laboratory. After a little experience the loss of animals from intubation by this method is all but eliminated.

METHOD

A No. 8 French rubber catheter with a depressed eye is used as the stomach tube. With the rat resting on the table and its head facing toward the operator's right, the left hand of the operator is spread across the animal; the thumb and index finger are so placed that the middle of the distal phalanx of the former is at the angle of the mouth, while the latter straddles the base of the skull of the animal (Fig. 1). This arrangement gives these two fingers control of the head. The palm of the left hand fixes the animal to the table by gentle pressure on its back. In this way all four feet of the animal are immobilized. The thumb is then slid upward and backward, a procedure which draws the tissues back, opens the jaws, and at the same time slightly turns the head of the animal toward the operator (Fig. 2). This position must be maintained until the end of the procedure. The tip of the rubber catheter is moistened with normal saline (not oil) and is inserted over the tongue and

From the Fels Research Institute, Temple University School of Medicine.
Received for publication, Sept. 19, 1946.

passed either along the right or the left side of the mouth, keeping the tube as close to the cheek as possible. By this maneuver the tube moves readily down the esophagus into the stomach (Fig. 3). If any resistance to the passage of

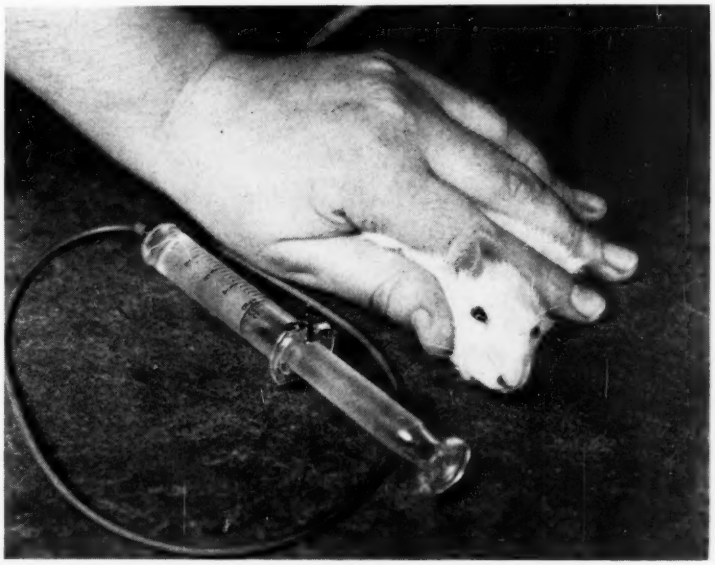

Fig. 1.

Fig. 2.

the tube is met, the tube should be removed and reintroduced. The length of catheter necessary to intubate the stomach depends upon the size of the animal. With a little experience, impingement of the tip of the tube upon the

stomach wall is readily recognized. When measured amounts of fluids are to be injected, the catheter is attached to a Luer syringe and the fluid is drawn through the catheter before being introduced into the stomach. This permits one to ignore the fluid which remains in the catheter at the end of the injection. After the fluid is slowly injected into the stomach, the catheter, with syringe attached, is quickly withdrawn.

Fig. 3.

We have intubated several thousand rats by this method. In some the catheter was inserted three times a day for several months without apparently causing any discomfort to the animal. The technique may be learned quickly, and after a little experience one operator can handle sixty animals an hour. We have used the method successfully on animals weighing as little as 60 grams each.

SUMMARY

A simple and safe method for gastric intubation and for the gastric instillation of fluids in the rat is described.

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BOOK REVIEWS

Human Embryology. By *Bradley M. Patten*, Ph.D., Professor of Anatomy, University of Michigan School, Ann Arbor, Michigan. The Blakiston Company, Philadelphia, 1946. Price $7.00. Cloth with 776 pages (illustrated).

The recent publication of the new textbook of *Human Embryology* by Professor B. M. Patten is an unusually serviceable and timely contribution to the study of the subject. For a textbook covering material so full of detail, little of importance has been omitted. The volume is admirable for the clarity with which it is written and is highly recommended. Because the book is scholarly and gives a somewhat dogmatic approach to the anatomy of the developing human embryo and fetus, it will probably be adopted as a standard textbook in many medical schools. The author does not stray from his purpose of designing the work primarily for students of medicine and for physicians. Embryology is presented in a practical manner to both student and teacher, on paper that is relatively good for these times. The compilation of subject matter is amazingly complete and accurate, and the writer includes few text references to disrupt the continuity of the discourse. However, a list of 939 articles is arranged according to subject matter in the bibliography. The index is both complete and easy to use.

While the aim of the book is both analytical and informative, it presupposes a general knowledge of the subject, particularly that of the development of the chick. Emphasis is given to the comprehension of developmental processes rather than to illustration of any particular form, other than that of man. Comparative anatomical aspects of the subject, therefore, are minimal. It is apparent, however, that the author is well aware that many features of human embryonic development are incomprehensible unless viewed in the light of development of some lower forms. The section on the phylogenetic development of muscles of facial expression, for example, is certainly adequate. The author's keen insight into the subject, appreciation of students' difficulties, and broad experience in interpreting our present knowledge of human developmental processes is apparent in the topics selected for detailed discussion.

Organization of subject matter is such that it provides a direct continuity between consecutive subjects, making for a unified discourse on human development rather than a series of individual treatises. Following the newer trend, proper names have been replaced largely by names descriptive of the structure; the term *ovum* is used only in the restricted sense. Beginning with a short account of the history of embryology and the purpose of embryology in the medical curriculum, the text passes to a logical consideration of male and female reproductive organs. The sexual cycle is discussed adequately in the chapter devoted to processes of fertilization and sex determination. Segmentation of the zygote and establishment of the germ layers and the early embryonic body are well described, especially the section on formation of mesoderm and the origin of the primitive streak. The Hertig-Rock 11- and 12-day-old human embryos are discussed, but the more recent 7- and 9-day-old human embryos are not included. Chapter five gives a good insight into the early differentiation of various regions of the body from primitive streak material. It is especially serviceable as a guide to the study of the 5 to 15 mm. pig embryo and is a good substitute for a separate section devoted to laboratory instructions. Of value also is a concise but pertinent account of early differentiation of the organ systems in very young human embryos which facilitates the comprehension of a complete picture of embryos, as a whole, up to six weeks of age, an age comparing favorably with that of 5 to 15 mm. pig embryos. Subsequent stages of growth of each organ system are considered in detail in later chapters. The sections on fetal membranes and the placenta, as well as those concerned with the organ systems, are well balanced and provide a good

embryologic foundation for advanced medical study. The chapter on age and body growth and form includes a number of authoritative graphs. Those depicting the growth of the body in stature during prenatal and postnatal life and mean weights of organs at different periods of fetal growth will be very useful. The description of twins, double monsters, and teratology leaves little to be desired. Causative factors operative in the production of developmental anomalies are also discussed. Particular stress is placed throughout the text on the more common developmental anomalies in human embryos. These are well described, admirably illustrated, and of value to clinicians. The section on Spina bifida is an excellent example, along with others cited, of the author's belief that it is difficult to try to explain all anomalies on the basis of the theory of developmental arrests.

Advanced stages of organogenesis, as well as histogenesis and frequent recourse to pictorial and descriptive accounts of postnatal development and comparisons between the newborn and adult, permeate the text. This highly desirable practice facilitates the correlation of embryology with the study of histology and gross anatomy. Functional aspects of the work are intimately associated with developing structures throughout the greater part of the book. Outstanding sections of the text are those dedicated to the connective tissues and skeleton, nervous system, body cavities and mesenteries, urogenital system, and postnatal changes in circulation. Emphasis is also ascribed to histogenesis of the cerebral cortex, retina, general sense organs, digestive system, endocrine organs, and gonads. Much of the illustrative data has been obtained from the University of Michigan slide collections. The numerous illustrations are well chosen and instructive. While they have been designed for more than sheer beauty, it would be difficult to surpass many of these illustrations in artistic merit. Illustrated gross dissections tell an accurate story and serve as excellent teaching guides. The figures showing postnatal development of the skeletal, muscular, nervous, digestive, and circulatory systems should be of great value to those less expert than the author.

The author clearly portrays embryology as a basis for understanding other anatomical sciences. He points out that it will lead the student "beyond anatomical memorizing to a comprehension of anatomy." It will be recognized that such a textbook, despite the advances of experimental embryology, must contain some degree of dogmatism. The author's experience and contributions, however, have made his opinions well worth knowing and worthy also of the dignified format chosen.

J. H. Van Dyke.

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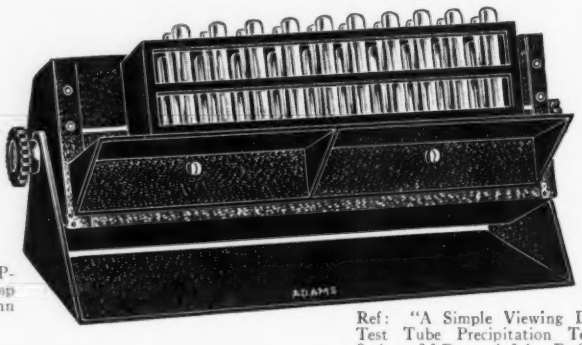

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Ref: "A Simple Viewing Device for Reading Test Tube Precipitation Tests," Howard P. Steiger, M.D., and John F. Simpson, Charlotte, N. C., The Journal of Venereal Disease Information, September, 1945.

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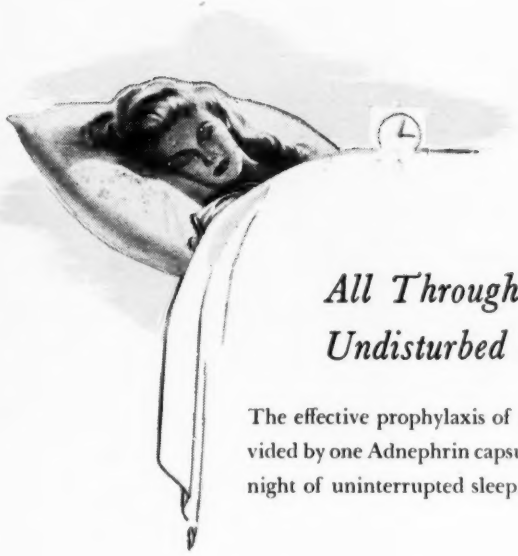

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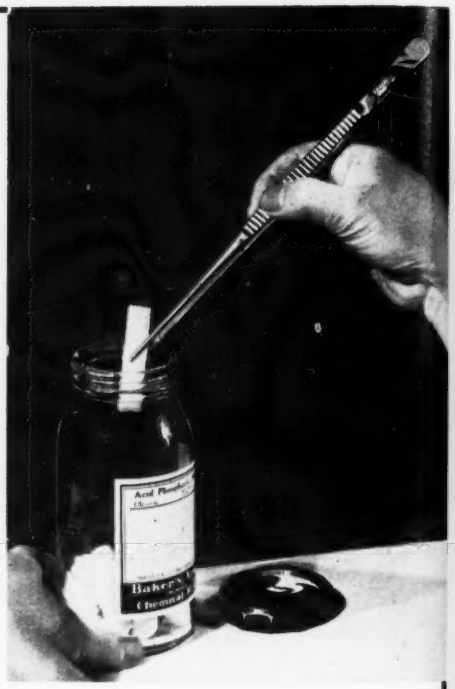

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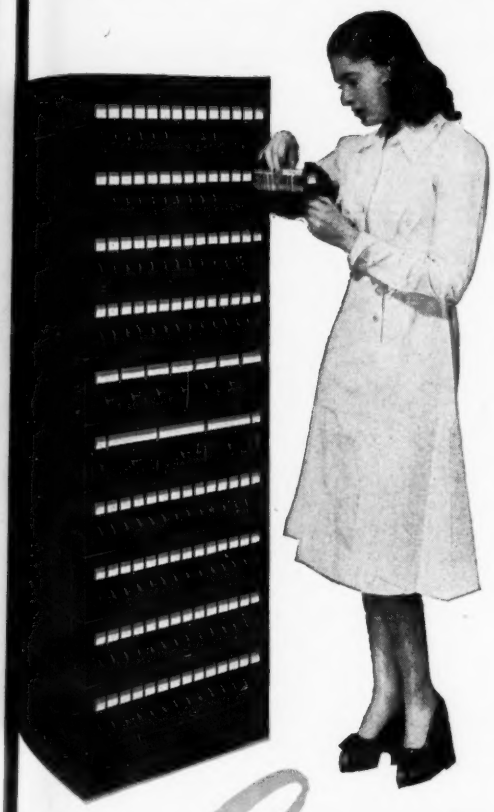

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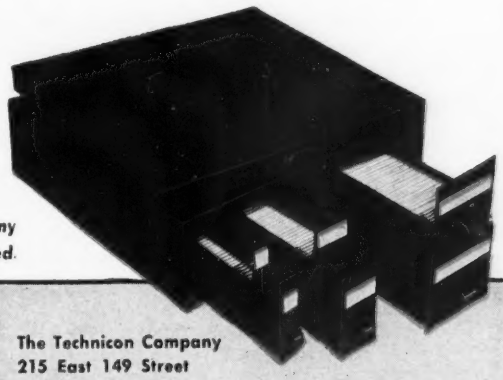

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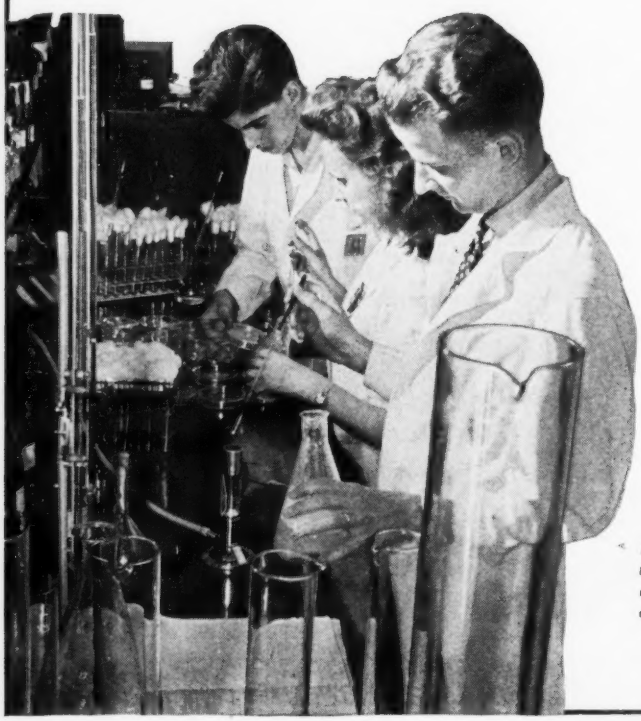

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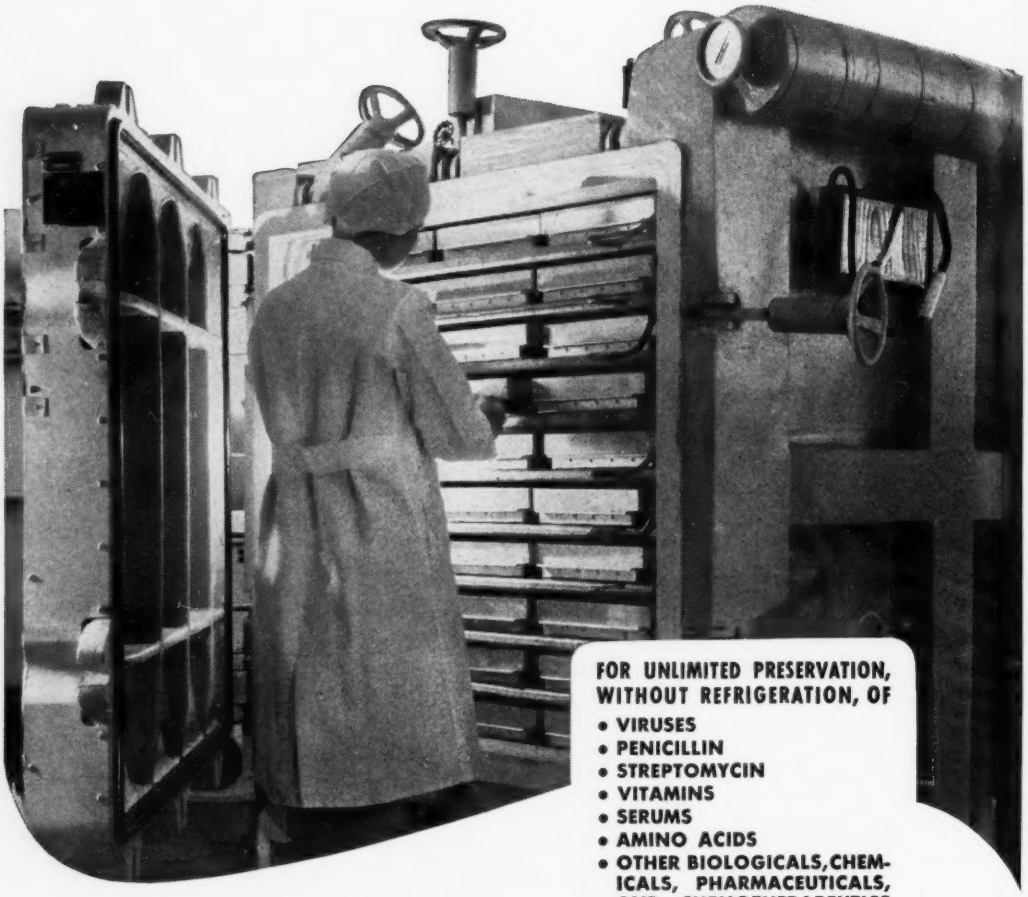

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* Journal of Immunology, Vol. 50. No. 1, 1945.

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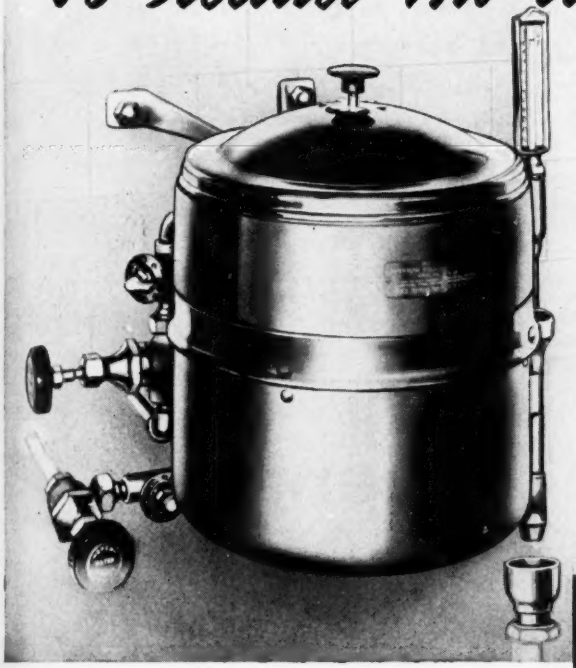

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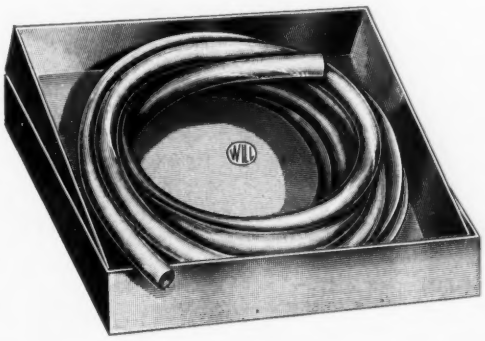

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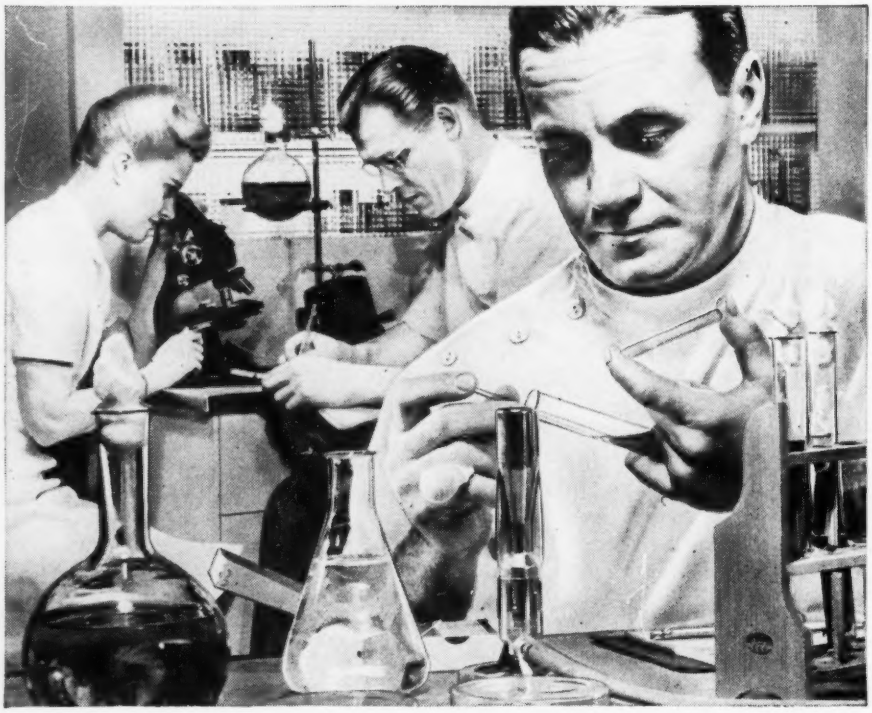

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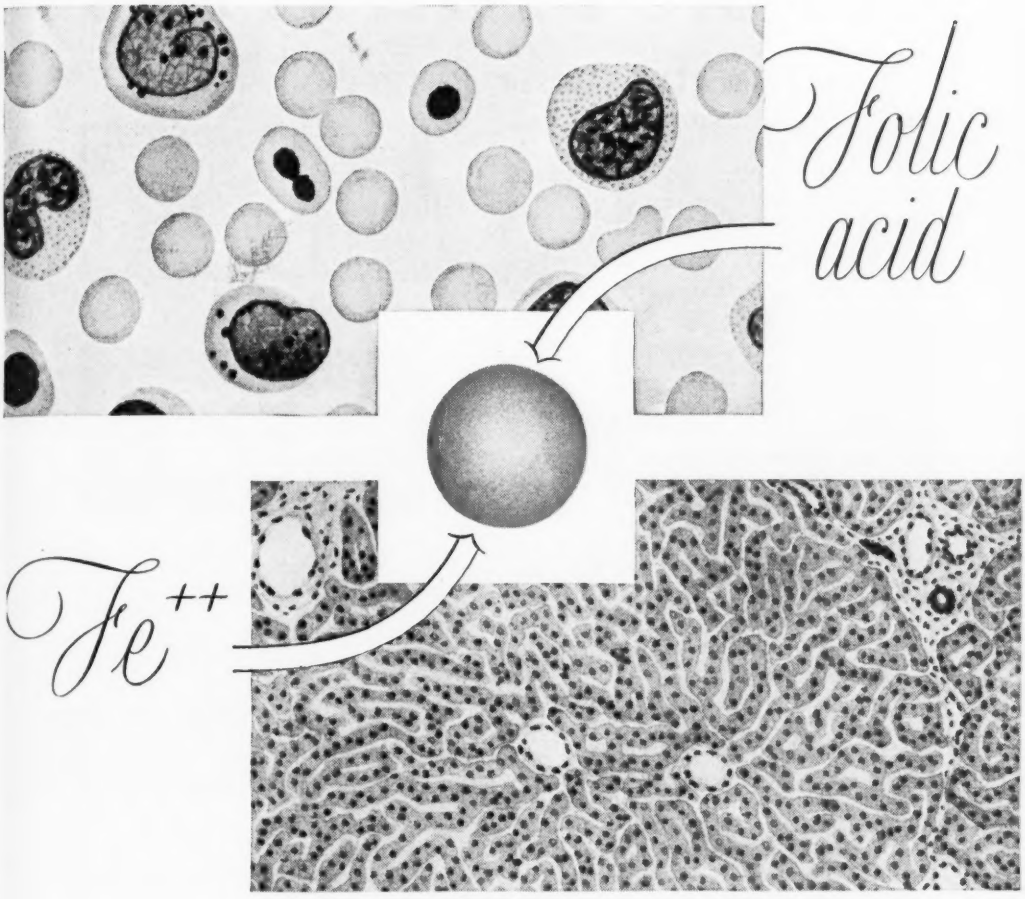

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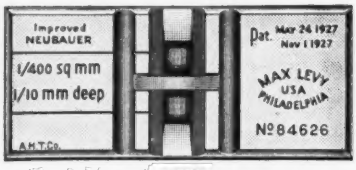
3369-F.



3301.

3319.

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Sharp, Deep Permanent Rulings, cut directly into the surface of the plane glass, which is not easily scratched. Lines are of high visibility when cell is filled with diluted blood.

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Deep, Wide Protecting Trenches, both outside and transverse, which facilitate cleaning.

Finding Lines on matte surfaces at left of slide which facilitate location of ruled area without danger of disturbing the distribution.

Green Glass. Specially selected to minimize eye fatigue. Rulings and corpuscles appear sharply defined against the subdued background.

3369-F. Hemacytometer Outfit, consisting of Levy Counting Chamber of colorless glass with double Improved Neubauer ruling and two cover glasses; two Thoma diluting pipettes, one for red and one for white corpuscles; in case of dark green artificial leather lined with velveteen 13.25

3369-G. Ditto, but with Levy-Hausser Counting Chamber with double Improved Neubauer ruling 17.25

NOTE—Above Hemacytometers can be supplied at same price with Counting Chambers of green glass in place of the colorless glass.

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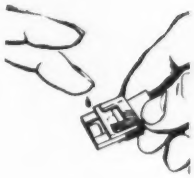
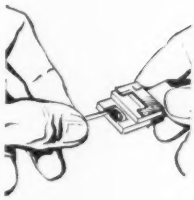
A Blood is dropped directly on glass chamber.



B Blood is quickly hemolyzed by agitating with hemolysis applicator.



C Chamber is inserted and the fields matched.

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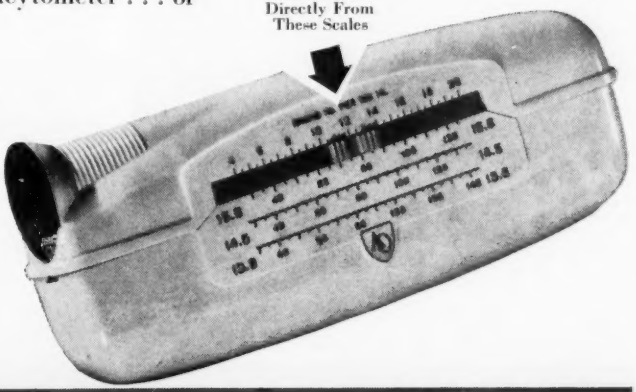

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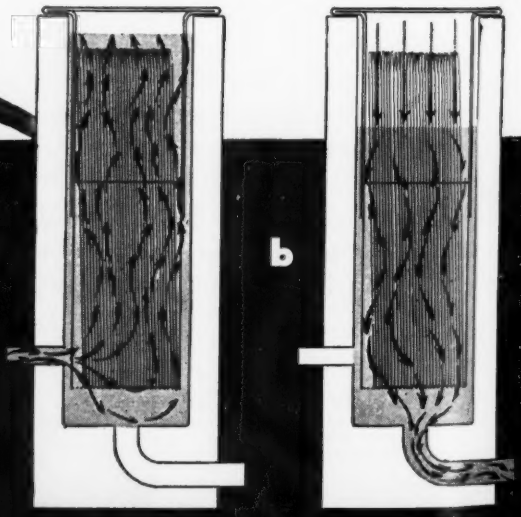

the efficient Technicon washing principle is graphically shown in these schematic views

here's how it works . . .

(a) Schematic view showing washer, loaded with pipettes, immersed in Technicon washing chamber. Water surges in at inlet, passing upward through open-mesh floor of rack, sweeping through pipettes, until it reaches top level shown.

(b) When water has reached top, the outlet valve opens. Water then flushes out rapidly, sucking through pipettes, and carrying away wastes and residues. Water is under pressure, in violent turbulence, during entire cycle.

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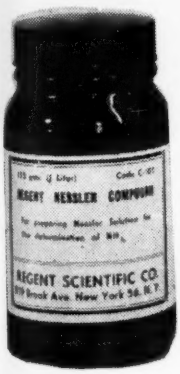

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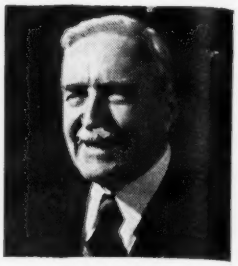

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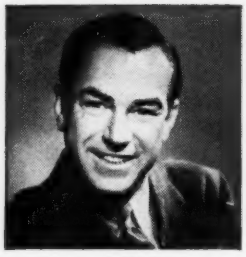

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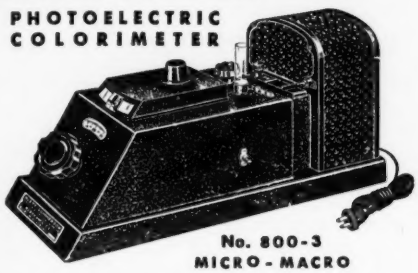

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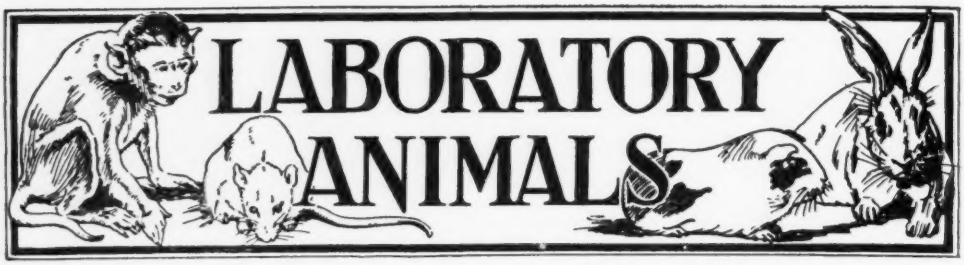

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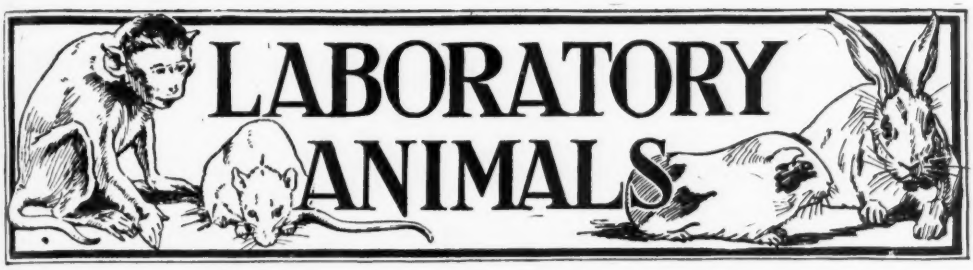

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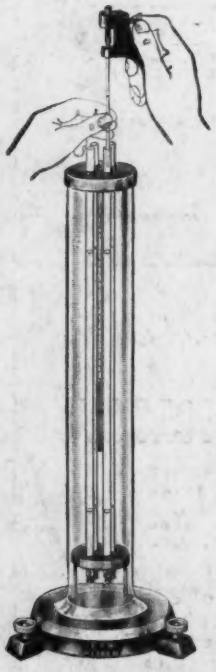

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